REMARKS/ARGUMENTS

Claims 1-10 are pending in the application. Claims 1-7 and 10 remain in the application. Claims 8-9 have been canceled as subject to a restriction requirement. Applicants reserve the right to seek any subject matter in claims 8-9 in related applications.

Claims 1, 3-7 and 10 stand rejected. Claim 2 stands objected to.

Restriction Requirement

Applicants confirm the election of Group I, Claims 1-7 and 10.

The Amendment

In the Claims

Claims 8-9 have been canceled as subject to a restriction requirement.

Claim 10 has been amended to clarify that the nucleic acid molecules in step (a) are those recited in claim 1. The language "that encode a polypeptide having PhzO activity" has been deleted from step a since this is inherent in the definition of the nucleic acid molecules of claim 1. Support for this amendment is found throughout applicants' specification (see, in particular, paragraph [0221]) and in original claim 1.

Rejection Under 35 U.S.C. 112

Claim 3 stands rejected under 35 U.S.C. 112, first paragraph as failing to comply with the enablement requirement. Applicants submit herewith a Declaration of Deposit of Biological Material wherein applicant Linda Thomashow states that plasmids identified below were introduced into the designated host strains and the transformed strains were deposited under terms of the Budapest Treaty with Agricultural Research Service Culture Collection (NRRL) National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604 USA on the date listed and given the following accession numbers:

Host	Plasmid	Accession No.	Date of Deposit
E. coli JM109	pUC2.9XP, clone 12	NRRL B-30341	10/06/2000
E. coli JM109	pUC2.9XP, clone 16	NRRL B-30342	10/06/2000

P. fluorescens 2-79	pUC2.9XP, clone 12	NRRL B-30343	10/06/2000
P. fluorescens 2-79	pUC2.9XP, clone 12	NRRL B-30344	10/06/2000
E. coli JM109	pGEM-PHZO	NRRL B-30345	10/11/2000
E. coli JM109	pGEM-PHZO	NRRL B-30346	10/11/2000

Copies of the NRRL Budapest Treaty Deposit Receipts and NRRL Budapest Treaty Viability Statements are attached to the Declaration of Deposit of Biological Material.

In view of the foregoing, it is submitted that claim 3 fully meets the requirements of 35 U.S.C. 112, first paragraph.

Rejection Under 35 U.S.C. 102/103

Claims 1, 4-7 and 10 stand rejected under 35 U.S.C. 102(b) as being anticipated by or, in the alternative, under 35 U.S.C. 103(a) as obvious over Ligon, et al. (A). In particular, the Examiner alleges that Ligon et al. (A) teaches in column 34, lines 56-60 that "[t]he shortest deletion derivative which was found to confer biosynthesis of all three phenazine compounds to *E. coli* contained an insert of approximately 6 kb and was designated pLSP18-6H3de13". The Examiner further states that in column 35, lines 1-11, it is taught that pLSP18-6H3de13 contains four ORFs and that ORF4 is SEQ ID NO:22. The Examiner also states that the brief description of the sequences in columns 5-6 discloses that SEQ ID NO:21 is the DNA sequence encoding the phz4 gene, and that the sequence search attached to the end of the reference discloses that SEQ ID NO:1 from residues 5-146 is 100% identical with SEQ ID NO:21 of the instant reference. Therefore, the instant reference would appear to meet the requirements of (e) of claim 1 that it is a "nucleic acid which hybridizes under medium or high stringency conditions with the nucleotide sequence of SEQ ID NO:1 from nucleotide 89 through nucleotide 1564...[and] encodes a polypeptide having PhzO activity." The Examiner also states that medium stringency allows many other nucleic acids to hybridize to the nucleic acid than one with 100% local identity and therefore many other references could be used to reject this claim under this limitation.

Argument

Applicants respectfully submit that claims 1, 4-7, and claim 10, as amended, define an invention which is patentable over and unobvious over the cited art for the reasons set forth in detail below. The Examiner is directed to the Delaney et al. reference (Delaney, S. M., D. V. Mavrodi, R. F. Bonsall, and L. S. Thomashow, 2001, "*phzO* a Gene for Biosynthesis of 2-Hydroxylated Phenazine Compounds in *Pseudomonas aureofaciens* 30-84" *Journal of Bacteriology* 183:318-327) (copy enclosed). On page 322, column 1, the reference states: "To determine the biosynthetic potential of *phzXYFABCD*, the core operon was cloned downstream of a *tac* promoter and transposed from pUT-Km/30-84 into the genomes of *P. fluorescens* 2-79, Q8r1-96, and M4-80R. Transposition in each recipient was confirmed by PCR with *phzXY*-specific primers. RP-HPLC revealed that all three transformed strains produced PCA but not 2-OH-PCA or 2-OH-PHZ (Table 2), indicating that the core genes from strain 30-84 do not contain the information necessary for the synthesis of the 2-hydroxyphenazines." (emphasis added). This result presented in the Delaney et al. paper shows that the genes (ABCD) described in Ligon were **not** sufficient to allow the production of hydroxyphenazine compounds.

On page 322, column 2 of the Delaney et al. paper, it states: "The *phzO* gene from *P. aureofaciens* 30-84 was cloned in pUCP26 under the control of the *lac* promoter and expressed in *E. coli* JM109. Cells from induced cultures expressing PhzO produced a unique band of approximately 55 kDa on SDS-polyacrylamide gels, in good agreement with the size predicted by nucleotide sequence analysis. Induced cultures of *E. coli* expressing PhzO, either in pUCP26 or in pGEM-T Easy, converted PCA (0.3 or 0.5 mg/ml in 5% $NaHCO_3$) to 2-OH-PCA and 2-OH-PHZ within 3 h, whereas no such conversion occurred in control cultures harboring only the respective vectors (Fig. 2). These results indicate that PhzO, independent of up- and downstream sequences, is sufficient to hydroxylate PCA." (Emphasis added).

On page 324, column 1 of Delaney et al., the Discussion section states: "Results of the current study show clearly that phzXYFABCD, the core phenazine biosynthetic operon of strain 30-84, is responsible only for the synthesis of PCA (Fig. 5). When transformed with these genes, the sole phenazine product synthesized by P. fluorescens strains Q8r1-96 and M4-80R (which themselves do not produce phenazines) was PCA."

The information cited from the Delaney et al. paper is also discussed by applicants in the instant application in paragraphs [02118] and [0219]. In view of the foregoing information, it is submitted that claims 1, 4-7, and 10 are not anticipated by or obvious in view of Ligon et al. (A).

The Examiner is also directed to the Mavrodi et al. reference (Mavrodi, D. V., V. N. Ksenzenko, R. F. Bonsall, R. J. Cook, A.M. Boronin, and L. S. Thomashow, 1998, "A Seven-Gene Locus for Synthesis of Phenazine-1-Carboxylic Acid by *Pseudomonas fluorescens* 2-79" *Journal of Bacteriology* 180(9):2541-2548) (copy enclosed): On page 2547, column 2, the authors state: "Moreover, our observations that expression in *E. coli* of incomplete loci lacking *phzAB* or *phzXY* yielded mixtures of compounds including PCA and unsubstituted phenazine, whereas expression of the complete locus from either strain enabled the synthesis of large amounts of essentially homogeneous PCA, argue that the compounds detected in earlier expression studies with strain 30-84 that included only *phzFABCD* are products of inefficient or nonspecific synthesis that do not accurately reflect the biosynthetic potential of the intact locus." (Emphasis added). In view of the foregoing information, it is further submitted that claims 1, 4-7, and 10 are not anticipated by or obvious in view of Ligon et al. (A).

phzO is recognized in the NCBI database as a member of the highly conserved *4-hydroxyphenylacetate 3-hydroxylase family.* However, the similarity begins at amino acid 135 - much further into the protein than what is in the Ligon clone. This is further evidence of the patentability of the claimed invention.

In view of the foregoing it is submitted claims 1, 4-7 and 10 are patentable over Ligon et al. as the cited art does not teach or render obvious an isolated nucleic acid molecule which encodes a polypeptide having PhzO activity. It is further submitted that the above information rebuts the Examiner's statement on page 4 of the Office Action that many other references could be used to reject this claim under this limitation.

Rejection Under 35 U.S.C. 103

Claims 10 stands rejected under 35 U.S.C. 103(a) allegedly as being unpatentable over Pierson et al. (U). The Examiner states that the reference teaches the location of the phenazine biosynthetic in the genome of *Pseudomonas aureofaciens*. In figure 3 it is shown that plasmids pLSP18-6 and pLSP18-6H3del3 both produce 2-hydroxyphenazine, which is the definition in the instant specification of "PhzO activity". The instant claim is not drawn to any particular sequence encoding PhzO activity but simply to "transforming a host with one or more nucleic acid molecules that encoding a polypeptide having PhzO activity". The Examiner states that it would have been obvious to one of ordinary skill in the art to produce a polypeptide having PhzO activity by transforming either of these plasmids into a host cell and grow the host cell, given the general knowledge concerning cloning of nucleic acids and making host cells, absent unexpected results.

Argument

Applicants respectfully submit that the claim 10, as amended, defines an invention which is unobvious over the cited art. Claim 1 has been amended to recite the limitations of claim 1. Pierson et al. does not render obvious the sequences of claim 1. The arguments set forth above with regard to claims 1, 4-7, and 10 regarding the Ligon et al. reference also apply to the Pierson et al. reference and are incorporated herein by reference. The statements discussed above from the Mavrodi and Delaney papers refute this Pierson figure completely. These constructs produce a mixture of heterocyclic compounds, not specifically hydroxyphenazines. It should be noted that the Ligon patent is based on this paper.

In view of the foregoing it is submitted claim 10, as amended, is patentable over Pierson et al.

Objection to Claim 2

The Examiner did not set forth specific reasons for his objection to claim 2 noted in part 7 of the Office Action Summary. Applicants submit that claim 2 meets the requirements for patentability. Alternatively, applicants request clarification of the objection to claim 2.

Summary and Conclusion

The foregoing amendments and remarks are being made to place the application in condition for allowance. Applicants respectfully request consideration and respectfully request that a timely Notice of Allowance be issued in this case. Should the Examiner find that an interview would be helpful to further prosecution of this application, he is invited to telephone the undersigned at his convenience.

Respectfully submitted,

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Enclosures:
Delaney et al., *Journal of Bacteriology* 183:318-327 (2001)
Mavrodi et al., *Journal of Bacteriology* 180(9):2541-2548 (1998)

Accompanying Enclosures:
Declaration of Deposit of Biological Material with attached NRRL Budapest Treaty Deposit Receipts and NRRL Budapest Treaty Viability Statements
Postcard Receipt

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phzO, a Gene for Biosynthesis of 2-Hydroxylated Phenazine Compounds in *Pseudomonas aureofaciens* 30-84

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Certain strains of root-colonizing fluorescent *Pseudomonas* spp. produce phenazines, a class of antifungal metabolites that can provide protection against various soilborne root pathogens. Despite the fact that the phenazine biosynthetic locus is highly conserved among fluorescent *Pseudomonas* spp., individual strains differ in the range of phenazine compounds they produce. This study focuses on the ability of *Pseudomonas aureofaciens* 30-84 to produce 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine from the common phenazine metabolite phenazine-1-carboxylic acid (PCA). *P. aureofaciens* 30-84 contains a novel gene located downstream from the core phenazine operon that encodes a 55-kDa aromatic monooxygenase responsible for the hydroxylation of PCA to produce 2-OH-PCA. Knowledge of the genes responsible for phenazine product specificity could ultimately reveal ways to manipulate organisms to produce multiple phenazines or novel phenazines not previously described.

Certain strains of root-colonizing fluorescent *Pseudomonas* spp. have gained attention in recent years because they produce broad-spectrum antibiotic metabolites that can provide protection against soilborne root diseases (46). One such class of antibiotics, the phenazines, encompasses a large family of heterocyclic nitrogen-containing compounds produced in late exponential and stationary phase. The ability to produce phenazines is limited almost exclusively to bacteria and has been reported in members of the genera *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* (48). There are currently over 50 known phenazine compounds with the same basic structure, differing only in the derivatization of the heterocyclic core. These modifications largely determine the physical properties of phenazines and influence their biological activity against plant and animal pathogens.

The broad-spectrum activity exhibited by phenazine compounds against fungi and other bacteria is not understood. It is thought that they diffuse across the membrane and, once inside the cell, accept a single electron, disrupting respiration by interfering with the normal process of electron transport. This results in the overproduction of O_2^- and H_2O_2, which overwhelm cellular superoxide dismutases and ultimately cause cell death. The cellular superoxide dismutases of *Pseudomonas aeruginosa*, a bacterium which produces the phenazine compound pyocyanin, are more active than those of phenazine-nonproducing bacteria such as *Escherichia coli*, and they provide protection against phenazines (18, 19).

Several studies conducted in the early 1970s revealed tight links between phenazine biosynthesis and the shikimic acid pathway (48), but the biochemistry and genetic control of phenazine synthesis are still not fully understood. Chorismic acid has long been recognized as the branch point from the shikimic acid pathway to phenazine synthesis (26). Studies with radiolabeled precursors suggest that the phenazine core is formed by the symmetrical condensation of two molecules of chorismic acid (7, 20, 22, 26), while the amide nitrogen of glutamine serves as the immediate source of nitrogen in the heterocyclic nucleus of phenazine compounds (36). Phenazine-1,6-dicarboxylic acid is the first phenazine formed, and it is thought to be converted to phenazine-1-carboxylic acid (PCA), a key intermediate in the synthesis of other phenazines by fluorescent pseudomonads (6, 20, 22, 28).

Genetic studies in fluorescent *Pseudomonas* spp., the only microorganisms for which the genes responsible for the assembly of the heterocyclic phenazine nucleus have been cloned and sequenced, support this model. The phenazine biosynthetic loci from *P. fluorescens* 2-79 (27), *P. aureofaciens* 30-84 (27, 33), *P. aeruginosa* PA01 (D. V. Mavrodi and L. S. Thomashow, unpublished data), and *P. chlororaphis* PCL1391 (T. F. C. Chin-A-Woeng, D. van den Broek, G. de Voer, K. M. G. M. van der Drift, J. E. Thomas-Oates, B. J. J. Lugtenberg, and G. V. Bloemberg, *Pseudomonas* '99: Biotechnology and Pathogenesis, abstr. S48, 1999) are highly conserved. Each contains a seven-gene core operon regulated in a cell density-dependent manner by homologues of LuxI and LuxR (25, 52; D. V. Mavrodi and S. K. Farrand, unpublished data). In *P. fluorescens* 2-79, *P. aureofaciens* 30-84, and *P. chlororaphis* PCL1391, the *phzI/R* genes are found directly upstream from the phenazine core. Phenazine production in *P. aeruginosa* is controlled by two sets of regulatory proteins, *rhlI/R* and *lasI/R*, located elsewhere in the genome. The core gene products PhzC, PhzD, and PhzE, which are homologous with PhzF, PhzA, and PhzB in strain 30-84, are similar to enzymes of shikimic and chorismic acid metabolism. Sequence comparisons of PhzD and

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PhzE with other chorismate-modifying enzymes have shed new light on probable intermediates in the PCA pathway, suggesting that phenazine synthesis proceeds via the intermediates aminodeoxyisochorismic acid and 3-hydroxyanthranilate (27) rather than anthranilate, as suggested previously (12).

Although the phenazine biosynthetic loci of fluorescent pseudomonads are highly homologous, individual species typically differ in the range of compounds they produce. Previous work by Pierson et al. (33) suggested that the *phzC* gene of *P. aureofaciens* 30-84, and in particular the last 28 amino acids of the PhzC protein, are essential for the production of 2-hydroxyphenazine-1-carboxylic acid (2-OH-PCA) and 2-hydroxyphenazine (2-OH-PHZ), derivatives that are characteristic of strains previously designated *P. aureofaciens* but now classified as *P. chlororaphis* (24). The purpose of the present study was to determine the genetic basis for the production of these hydroxyphenazines by *P. aureofaciens* 30-84. Two possibilities were considered: first, that product specificity is determined by amino acid substitutions within the core biosynthetic genes, as suggested previously (33); or second, that a core pathway conserved among fluorescent pseudomonads is responsible for the synthesis of PCA, which then can be modified in a strain- or species-specific manner to yield a variety of different phenazine products. Knowledge of the mechanisms responsible for phenazine product specificity ultimately could reveal ways to manipulate organisms to produce multiple phenazines or hybrid phenazine products not previously described. Such compounds may have improved activity against soilborne plant pathogens or may lead to the development of novel pharmaceutical products.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Rifampin-resistant derivatives of *P. fluorescens* strains 2-79, M4-80R, and Q8r1-96 and *P. chlororaphis* 30-84 (referred to here by its original designation, *P. aureofaciens* 30-84) were used. *Pseudomonas* strains were grown at 28°C in Luria-Bertani (LB) broth, 2× YT broth (37), or M9 minimal medium (2) supplemented with sodium citrate to a final concentration of 40 mM as a carbon source. *E. coli* strains were grown in LB broth or 2× YT broth at 28 or 37°C. To enhance phenazine production, *Pseudomonas* strains were grown in LB broth supplemented with 1.5% glucose. When appropriate, antibiotic supplements were used at the following concentrations: tetracycline, 12.5 μg/ml (*E. coli*) or 25 μg/ml (*Pseudomonas* strains); rifampin, 100 μg/ml; kanamycin, 100 μg/ml; neomycin, 100 μg/ml (*P. fluorescens* 2-79); chloramphenicol, 35 μg/ml; and ampicillin, 100 μg/ml.

DNA manipulations. Standard methods were used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and ligation (2). *Pseudomonas* and *E. coli* cells were transformed by electroporation in a Gene Pulser II system (Bio-Rad, Hercules, Calif.) according to the method of Enderle and Farwell (11) at settings of 25 μF for the capacitor, 200 Ω resistance, and an electric field of 1.8 kV/cm. Genomic DNA was isolated and purified by a cetyltrimethylammonium bromide (CTAB) miniprep procedure (2). For Southern blotting and hybridization, 500 ng of genomic DNA was digested with *Eco*RI and *Pst*I, separated by electrophoresis in an 0.8% agarose gel, and transferred onto a BrightStar-Plus nylon membrane (Ambion, Inc., Austin, Tex.) in 0.4 M NaOH with subsequent cross-linking by exposure to UV irradiation (2). Membranes were prehybridized for 3 h at 60°C in a solution containing 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 4× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), and 250 mg of denatured salmon sperm DNA/ml. After prehybridization, the membranes were incubated with specific probes overnight under the same conditions and washed with 2× SSC and 0.1% SDS at room temperature, 0.2× SSC and 0.1% SDS at room temperature, 0.2× SSC and 0.1% SDS at 60°C, and 0.1× SSC and 0.1% SDS at 60°C. DNA-DNA hybrids were detected with the BrightStar non-isotopic detection kit (Ambion Inc.) according to the manufacturer's protocol. The 2.1-kb *phzO* probe was amplified by PCR from *P. aureofaciens* 30-84 genomic DNA with the oligonucleotide primers 30-84XBA (5'-AAG TCC AGA TGC GAA AGA ACG-3') and PHZO10 (5'-AAG TGG CAT GGC TCG AAC AAA G-3'). Amplification was carried out in a 25-μl reaction mixture containing 1× thermophilic DNA polymerase buffer (Promega Corp., Madison, Wis.), 1.5 mM $MgCl_2$, 5.0% (final concentration) dimethyl sulfoxide (Sigma Chemical Co., St. Louis, Mo.), 200-μM concentrations of dGTP, dATP, dCTP, and dTTP (Perkin-Elmer, Norwalk, Conn.), 20 pM of each primer, and 1.2 U of *Taq* DNA polymerase (Promega Corp.). Amplifications were performed with a PTC-200 thermal cycler (MJ Research Inc., Watertown, Mass.). The cycling program included a 45-s initial denaturation at 94°C followed by 30 cycles of 94°C for 45 s, 51°C for 45 s, and 72°C for 1.5 min. Amplified DNA was labeled with a random primer biotin labeling kit (NEN Life Science Products Inc., Boston, Mass.).

DNA sequencing and analysis. DNA was sequenced by using the ABI Prism Dye Terminator Cycle sequencing kit (Perkin-Elmer), according to the manufacturer's instructions. All custom-designed oligonucleotides came from Operon Technologies Inc. (Alameda, Calif.). Sequence data were compiled and analyzed for open reading frames and codon usage with the Omiga version 1.1.3 software package (Oxford Molecular Ltd., Oxford, United Kingdom). A database search for similar protein sequences was carried out with the BLAST (44) and FASTA network servers at the National Center for Biotechnology Information and the European Molecular Biology Laboratory (EMBL), respectively. The probable domain homologies search was performed with PROSITE (EMBL, Heidelberg, Germany) (3) and ISREC ProfileScan (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland [www.isrec.isb-sib.ch/software/PFSCAN_form.html]) computer services. The significance of the similarity of a predicted protein to known proteins was determined by calculating the binary comparison score (*Z* score). Pairwise alignments were obtained by using the BESTFIT program from the Wisconsin Package (Genetics Computer Group, Madison, Wis.), and the resulting percent identities, percent similarities, alignment scores (*A*), mean random alignment scores (*R*), and standard deviations (SD) ($n = 100$) were noted. *Z* scores were then calculated by the equation $Z = (A - R)/SD$. Multiple sequence alignments were built with Omiga's ClustalW and analyzed with the TreeView version 1.5.0 software package (31).

Mating and screening of transconjugants. Plasmids were mobilized from the donor strain *E. coli* S-17 (λ-*pir*) into *Pseudomonas* recipients by using the filter mating technique described by van Overbeek (49). To counterselect *E. coli* donor cells, mating mixtures were plated on M9 agar supplemented with appropriate antibiotics and sodium citrate as a carbon source. Positive isolates were replated and screened for the presence of phenazine genes by PCR with primers PHZ1 and PHZ2. The oligonucleotide primers PHZ1 (5'-GGC GAC ATG GTC AAC GG-3') and PHZ2 (5'-CGG CTG GCG GCG TAT TC-3') were used as universal phenazine primers to amplify a 1.4-kb fragment containing parts of *phzF* and *phzA* in *P. aureofaciens* 30-84, which correspond to *phzC* and *phzD* in *P. fluorescens* 2-79. The amplification was carried out in a 15-μl reaction mixture. The cycling program included an initial denaturation for 2 min at 94°C followed by 25 cycles of 94°C for 1 min, 56°C for 45 s, 72°C for 1.75 min, and a final extension at 75°C for 1 min. The oligonucleotide primers PHZX (5'-TTT TTT CAT ATG CCT GCT TCG CTT TC-3') and PHZY (5'-TTT GGA TCC TTA AGT TGG AAT GCC TCC G-3') were used to distinguish between the phenazine operons of *P. aureofaciens* 30-84 and *P. fluorescens* 2-79. These primers amplify a 1.1-kb DNA fragment containing parts of *phzX* and *phzY* from strain 30-84 but not from the corresponding, homologous *phzA* and *phzB* sequences of strain 2-79. The program included an initial denaturation at 94°C for 1.5 min followed by 30 cycles of 94°C for 45 s, 58°C for 30 s, 72°C for 1.75 min, and a final extension at 72°C for 1 min.

Protein expression. The *phzO* gene was cloned from *P. aureofaciens* 30-84 and expressed under the control of the *lac* promoter in the plasmid vector pUCP26. *E. coli* JM109 harboring pUCP26, pUCP2.9XP, or pUCP4.5 was grown in LB broth to an optical density at 600 nm of 0.6 and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 3 h later and total cellular protein was analyzed by electrophoresis in an SDS–10% polyacrylamide gel as described by Copeland (9). Alternatively, *phzO* was amplified by PCR from *P. aureofaciens* 30-84 genomic DNA with the primers PHZOstart (5'-CGA CTC TAG AAC GTT GTC CTT GAC C-3') and PHZO10 in a 30-μl reaction mixture with a cycling program that included a 45-s initial denaturation at 94°C and 29 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 3.25 min. The 1.8-kb reaction product was ligated into pGEM-T Easy (Promega) to give pGEM-PHZO, which was transformed into *E. coli* JM109. The resulting plasmid, pGEM-PHZO, contained the entire *phzO* gene preceded by 88 bp upstream of the start codon and 265 bp downstream of the coding sequence. Expression was induced as described above.

PCA transformation assay. *E. coli* JM109 bearing pUCP26, pUCP2.9XP, pGEM-T Easy, or pGEM-PHZO was grown at 37°C in 2× YT supplemented

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
P. aeruginosa		
PAO1	Phz$^+$, produces pyocyanin	12
ATCC 25007	Phz$^+$, produces pyocyanin, aeruginosins A and B	40
ATCC 25011	Phz$^+$, produces aeruginosins A and B	53
KN-1	Phz$^+$, produces pyocyanin	Laboratory collection
KNP-1	Phz$^+$, produces pyocyanin	Laboratory collection
KNP-2	Phz$^+$, produces pyocyanin	Laboratory collection
P. aureofaciens		
30-84	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	32
30-84mxO	Phz$^+$, produces PCA	This study
ATCC 13985	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	40
BS 1391	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	V. Kotchetkov
BS 1393	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	V. Kotchetkov
PGS12	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	15
AP-9	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	1
TX-1	Phz$^+$, produces PCA, 2-OH-PCA, and 2-OH-PHZ	EcoSoil Systems
P. chlororaphis		
ATCC 17411	Phz$^+$, produces chlororaphin	48
ATCC 17809	Phz$^+$, produces chlororaphin	ATCC
ATCC 9446	Phz$^+$, produces chlororaphin	40
P. fluorescens		
2-79	Phz$^+$ Rif^r, produces PCA	50
M4-80R	Phz$^-$ Rif^r	17
Q8r1-96	Phl$^+$ Rif^r	35
UQ 112	Phz$^+$, produces PCA	G. Botelho
UN 15	Phz$^+$, produces PCA	G. Botelho
UN 4127	Phz$^+$, produces PCA	G. Botelho
E. coli		
SM17 (λ-pir)	*thi pro hsdR hsdM recA rpsL* RP4-2 Tet^r::Mu Kan^r::Tn7	39
JM109	F′ *traD36 proA$^+$ proB$^+$ lacI lacZ*ΔM15/*recA1*	Promega Corp.
Plasmids		
pALTER-*Ex*1	ColE1 Tet^r SP6, *tac*, and T7 promoters	Promega Corp.
pNOT19	ColE1 *bla* accessory plasmid	38
pUCP26	pUCP18-derived broad-host-range vector Tet^r	51
pMOB3	Kan^r *cat sac*BR	38
pUCP4.5	pUCP26 containing 4.5 kb-*Xba*I-*Eco*RI DNA fragment from *P. aureofaciens* 30-84; Tet^r	This study
pUCP2.9XP	pUCP26 containing 2.9-kb *Xba*I-*Pst*I DNA fragment from *P. aureofaciens* 30-84; contains *phzO*, Tet^r	This study
pGEM-T Easy	Amp^r, pUC18-derived SP6 and T7 promoters, f1 ori, *lacZ*α	Promega Corp.
pGEM-PHZO	pGEM-T Easy containing 1.8-kb *phzO* fragment amplified from *P. aureofaciens* 30-84 by PCR	This study
p18Sfi	Amp^r, pUCP18-derived vector with *Sfi*I-*Eco*RI-*Sal*I-*Hin*dIII-*Sfi*I as multiple cloning site	21
pUT-Km-Tn*5*	Amp^r, Tn*5*-based delivery plasmid with Kan^r	21
pUT-Km/30-84	pUT-Km-Tn*5* with *phzXYFABCD* from *P. aureofaciens* 30-84 cloned between *Sfi*I sites	This study
pLAFR3	*IncP* Tet^r *cos$^+$ rlx$^+$*	41
pLSP259	pLAFR3 containing 20.9-kb DNA fragment from *P. aureofaciens* 30-84; Tet^r	32
pLSP282	pLAFR3 containing 24.7-kb DNA fragment from *P. aureofaciens* 30-84; Tet^r	32
pLSP282Δ20-9	pLAFR3 containing 15.0-kb DNA fragment from *P. aureofaciens* 30-84; Tet^r	32
pLSP282Δ30-8	pLAFR3 containing 11.2-kb DNA fragment from *P. aureofaciens* 30-84; Tet^r	32
pNOT2.9T-1	pNOT19 containing 2.9-kb *Xba*I-*Pst*I DNA fragment from *P. aureofaciens* 30-84; Tet resistance gene inserted at *Nco*I in same orientation as *phzO*; Tet^r Amp^r	This study
pOT-1	2.5-kb *Eco*RI-*Not*I DNA fragment from pNOT2.9T-1 in pNOT19; *phzO$^+$* Tet^r Amp^r	This study
pOT1-1	pOT-1 containing 5.6-kb *sac* genes from pMOB3; *phzO$^+$* Tet^r Amp^r Chl^r	This study

[a] Phz$^{+/-}$, the strain does (+) or does not (−) produce phenazines; Phl$^+$, production of 2,4-diacetylphloroglucinol; *bla*, β-lactamase; *cat*, chloramphenicol acetyltransferase; Amp^r, ampicillin resistance; Chl^r, chloramphenicol resistance; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Tet^r, tetracycline resistance.

with tetracycline. The cells were harvested, suspended in fresh medium, and induced with 0.5 mM IPTG. PCA was added to a final concentration of 0.3 or 0.5 mg/ml from a 25 mM stock solution in 5% (wt/vol) $NaHCO_3$. Samples were taken at 3-h intervals, extracted, and analyzed for phenazine composition by reverse-phase high-performance liquid chromatography (RP-HPLC).

Gene replacement mutagenesis of *phzO*. A *phzO* knockout mutant of *P. aureofaciens* 30-84 was generated by gene replacement as described by Schweizer (38). Briefly, a 2.5-kb *Pvu*II fragment bearing a tetracycline resistance gene from pALTER-*Ex*1 (Promega) was inserted into *phzO* at the *Nco*I site. The interrupted gene was subcloned into pNOT19, yielding pOT1, which was digested with *Not*I and ligated with a 5.3-kb pMOB3 *sac*B cassette. The resulting plasmid, pOT1-1, was mobilized into *P. aureofaciens* 30-84 from *E. coli* S-17 (λ-*pir*), and double crossover progeny were selected as described previously (38).

Analysis of phenazine compounds. Phenazine compounds were extracted according to the method of Bonsall et al. (5). Bacterial strains were cultivated for 72 h in LB broth supplemented with 1.5% glucose. The cultures were acidified

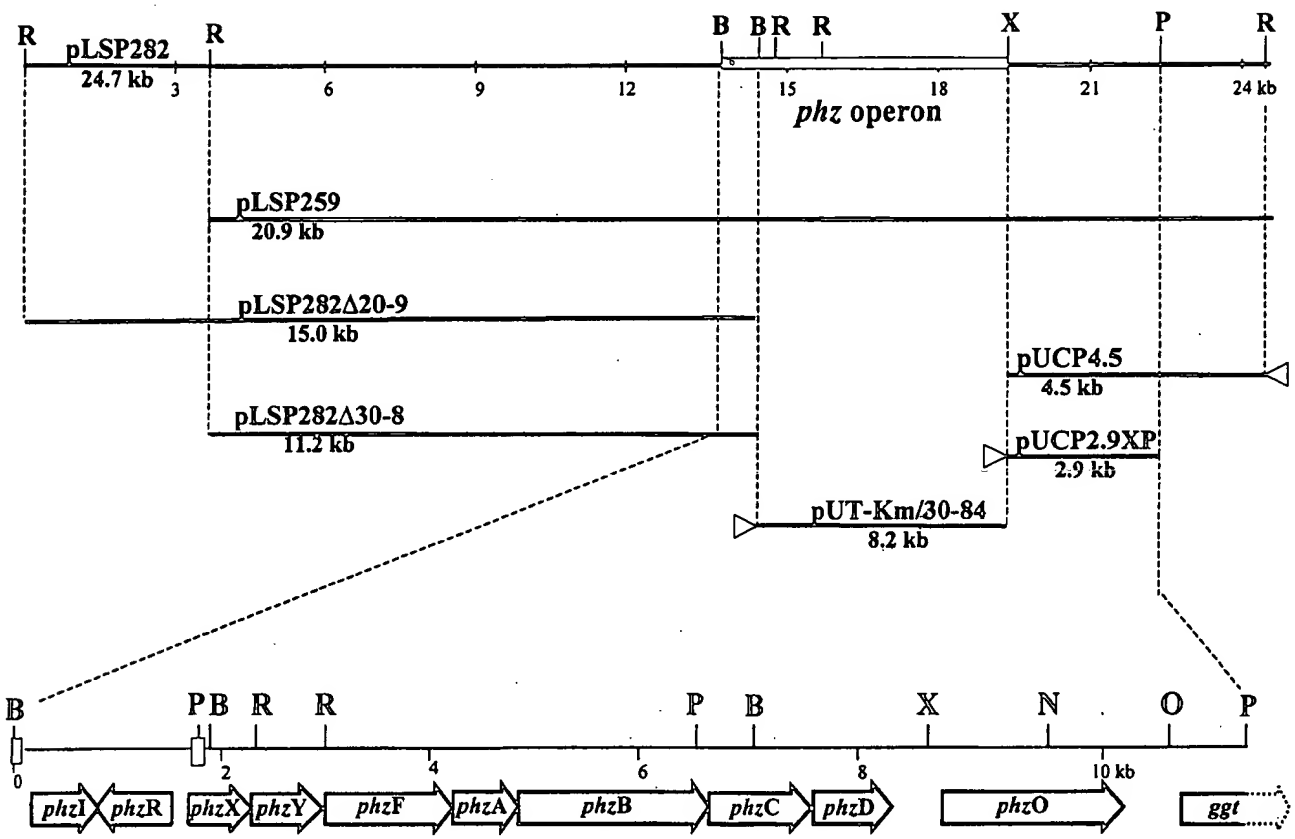

FIG. 1. Physical map of the constructs used in this study and description of the *phz* operon (*phzIRXYFABCD*) and *phzO* region in *P. aureofaciens* 30-84. The phenazine operon has been described previously (33). The *lux* boxes upstream of *phzI* and *phzX* are represented by □. ▷ indicates the orientation of the *lac* promoters in pUT-Km and pUCP26. *ggt* indicates the position of an open reading frame with similarity to gamma-glutamyl transpeptidase. The restriction enzymes indicated on the map are B, *Bgl*II; N, *Nco*I; O, *Not*I; P, *Pst*I; R, *Eco*RI; and X, *Xba*I.

with 10% trifluoroacetic acid (TFA) and then extracted twice with ethyl acetate. The organic phase containing the phenazines was evaporated to dryness and suspended in 35% acetonitrile (ACN)–0.1% TFA.

Since phenazine-producing *Pseudomonas* spp. often produce mixtures of phenazine compounds, a generalized HPLC protocol for detection of these metabolites was developed. The protocol utilized a NOVA-PAK C_{18} reverse-phase Radial-PAK cartridge (4 μm, 8 by 100 mm) (Waters Corp., Milford, Mass.) and solvent conditions consisting of a 2-min initial wash with 35% ACN–0.1% TFA in H_2O followed by a 25-min linear gradient to 100% ACN–0.1% TFA at a flow rate of 1.0 ml/min. The Waters HPLC system included a 710B WISP, 510 pumps, and a 680 automated gradient controller with a 990 photodiode array detector (Waters Corp.). Phenazine compounds were identified by retention time and UV spectrum. Standards included compounds purified from well-characterized strains (27, 32) and chemically synthesized compounds (PCA, 2-OH-PCA, and 2-OH-PHZ) obtained from Colour Your Enzyme (Bath, Ontario, Canada). Although the protocol allowed simultaneous identification of phenazine compounds including unsubstituted phenazine, PCA, 2-OH-PCA, 2-OH-PHZ, chlororaphin, and 1-OH-PCA, it failed to clearly separate PCA and 2-OH-PCA. 2-OH-PHZ, which is formed by spontaneous decarboxylation of 2-OH-PCA (see below), was therefore used as an indicator of 2-hydroxyphenazine synthesis, and when necessary, the presence of 2-OH-PCA in samples containing PCA was determined by peak purity and spectral analyses using the Waters 991 photodiode array (Waters Corp.).

Fungal inhibition assay. The inhibition of hyphal growth of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas* spp. strains 30-84, 30-84mxO, 2-79, and 2-79 harboring pUCP2.9XP was assayed as described by Ownley et al. (30) using Kanner agar supplemented with potato extract (KMPE), which supports the production of phenazine compounds. Plates were incubated at room temperature in the dark and radial growth of the fungus was measured after 5 days. The experiment was repeated twice with 7 or 8 replicates each time. Inhibition of mean fungal radial growth by each bacterial strain was analyzed for significance by the Student *t* test at a *P* level of 0.05.

Nucleotide sequence accession number. The nucleotide sequence for the *phzO* gene has been deposited in GenBank under accession number AF230879.

RESULTS

Localization of the 2-hydroxyphenazine gene. Previous studies by Pierson and Thomashow (32) identified two cosmids, pLSP259 and pLSP282, from a genomic library of *P. aureofaciens* 30-84 that were able to restore Phz^- mutants of 30-84 to production of PCA, 2-OH-PCA, and 2-OH-PHZ. These cosmids contain identical 11.2- and 9.2-kb *Eco*RI fragments, and an additional 3.8-kb *Eco*RI fragment is present in pLSP282 (Fig. 1). To determine whether pLSP259 and pLSP282 are sufficient to enable the synthesis of PCA, the two hydroxyphenazines, or all three products, each cosmid was introduced into *P. fluorescens* strains 2-79, M4-80R, and Q8r1-96 and the cosmid's presence was confirmed by PCR with *phzXY*-specific primers. Phenazine compounds produced by the transformed strains were extracted and analyzed by RP-HPLC. Transformants of all three strains harboring either cosmid produced both PCA and the 2-hydroxyphenazines (Table 2), indicating that pLSP259 and pLSP282 contain the necessary information required for the synthesis of all three compounds.

TABLE 2. Phenazine production as the result of introduction of plasmids into *P. fluorescens* strains

Plasmid	Phenazine compound	Result in recipient *P. fluorescens* strain:		
		2-79	Q8r1-96	M4-80R
pLSP282	PCA	+	+	+
	2-OH-PHZ	+	+	+
pLSP259	PCA	+	+	+
	2-OH-PHZ	+	+	+
pUT-Km30-84	PCA	+	+	+
	2-OH-PHZ	−	−	−
pLSP282Δ20-9	PCA	+	N/A[a]	N/A
	2-OH-PHZ	−	N/A	N/A
pLSP282Δ30-8	PCA	+	N/A	N/A
	2-OH-PHZ	−	N/A	N/A
pUCP4.5	PCA	+	N/A	N/A
	2-OH-PHZ	−	N/A	N/A
pUCP2.9	PCA	+	N/A	N/A
	2-OH-PHZ	+	N/A	N/A

[a] N/A, not analyzed.

We next determined whether the core phenazine operon, *phzXYFABCD*, which was present in both cosmids, was sufficient for the synthesis of the three phenazine products. Previous studies (33) had suggested that in *P. aureofaciens* 30-84, the C-terminal 28 amino acids of PhzC were necessary for the synthesis of 2-OH-PCA and 2-OH-PHZ. PhzC is a 278-amino acid, 30.3-kDa protein with 94% amino acid sequence identity to PhzF from *P. fluorescens* 2-79. These proteins have no common motifs or other similarities with other proteins of known function, but PhzF is absolutely required for the synthesis of PCA in strain 2-79 (27). A pairwise alignment of the terminal 28 amino acids of the two proteins revealed three conservative substitutions: lysine at position 251 in strain 30-84 instead of arginine in strain 2-79; glutamic acid at position 257 instead of aspartic acid; and valine at position 269 instead of isoleucine. To determine the biosynthetic potential of *phzXYFABCD*, the core operon was cloned downstream of a *tac* promoter and transposed from pUT-Km/30-84 into the genomes of *P. fluorescens* 2-79, Q8r1-96, and M4-80R. Transposition in each recipient was confirmed by PCR with *phzXY*-specific primers. RP-HPLC revealed that all three transformed strains produced PCA but not 2-OH-PCA or 2-OH-PHZ (Table 2), indicating that the core genes from strain 30-84 do not contain the information necessary for the synthesis of the 2-hydroxyphenazines.

The regions upstream and downstream of *phzXYFABCD* were next analyzed for genes enabling the conversion of PCA to 2-hydroxyphenazine derivatives. *P. fluorescens* 2-79 harboring either pLSP282Δ20-9, containing the 3.8- and 11.2-kb *Eco*RI fragments 5′ to the *phz* operon, or pLSP282Δ30-8, containing the 11.2-kb fragment (32) (Fig. 1), produced only PCA (Table 2), suggesting that the genes required for 2-hydroxyphenazine synthesis do not reside upstream of the core locus. The remaining 4.5-kb fragment downstream of the phenazine operon in pLSP282 was cloned into the broad-host-range vector pUCP26. A smaller 2.9-kb *Xba*I-*Pst*I fragment also was cloned into pUCP26 in the opposite orientation. Plasmid pUCP2.9XP contained the C-terminal region of *phzD* and downstream sequences under the control of the vector's *lac* promoter. Both plasmids were introduced into *P. fluorescens* 2-79 and the phenazines were extracted for RP-HPLC analysis. Strain 2-79 containing pUCP2.9XP, but not pUCP4.5, produced 2-OH-PHZ in addition to the PCA (Fig. 2), indicating that the 2.9-kb DNA fragment lacked a promoter, was colinear with the phenazine biosynthetic locus, and contained the gene(s) required for the conversion of PCA.

DNA sequence analysis. The 2.9-kb *Xba*I-*Pst*I fragment from *P. aureofaciens* 30-84 was sequenced in both directions and compiled with Omiga. Computer analysis revealed a large open reading frame, designated *phzO*, located 271 nucleotides downstream from *phzD* and preceded by a well-conserved ribosome binding site, GAGG. *phzO* encoded a 491-amino acid protein with a calculated molecular mass of 55.1 kDa. Homology searches with the deduced amino acid sequence revealed similarity to bacterial aromatic hydroxylases and monooxygenases (Table 3). Phylogenetic analysis of these aligned protein sequences resulted in the tree shown in Fig. 3. The high bootstrap values (from 1,000 resamplings) showed the robustness of these groups. A second open reading frame with the initiation codon GTG, preceded by the ribosome binding site GGAG and encoding a putative polypeptide with significant similarity (BLAST values of $6.6e^{-43}$) to gamma-glutamyl transpeptidase enzyme precursor proteins, was identified 656 bp downstream of the *phzO* termination codon.

Expression and functional analysis of PhzO. The *phzO* gene from *P. aureofaciens* 30-84 was cloned in pUCP26 under the control of the *lac* promoter and expressed in *E. coli* JM109. Cells from induced cultures expressing PhzO produced a unique band of approximately 55 kDa on SDS-polyacrylamide gels, in good agreement with the size predicted by nucleotide sequence analysis. Induced cultures of *E. coli* expressing PhzO, either in pUCP26 or in pGEM-T Easy, converted PCA (0.3 or 0.5 mg/ml in 5% $NaHCO_3$) to 2-OH-PCA and 2-OH-PHZ within 3 h, whereas no such conversion occurred in control cultures harboring only the respective vectors (Fig. 2). These results indicate that PhzO, independent of up- and downstream sequences, is sufficient to hydroxylate PCA. To determine whether PhzO is responsible for this reaction in *P. aureofaciens* 30-84, a tetracycline resistance gene was inserted into *phzO* and introduced in the genome by homologous recombination. *P. aureofaciens* 30-84mxO produced PCA but not 2-OH-PCA and 2-OH-PHZ. Finally, to test the hypothesis that the conversion of 2-OH-PCA to 2-OH-PZ occurs spontaneously in the absence of enzymatic activity, as suggested previously (13), solutions of synthetic 2-OH-PCA were incubated for 18 h in 0.1 M sodium phosphate buffer at pHs of 4.0, 6.0, 7.0, and 8.0, extracted, and analyzed by RP-HPLC. At pH 4.0, 2-OH-PZ accounted for only 0.2% of the total phenazine present after 18 h, but at pHs of 6.0, 7.0, and 8.0, 33.3, 74, and 64% of the 2-OH-PCA initially present was converted to 2-OH-PZ.

Conservation of PhzO among phenazine-producing fluorescent *Pseudomonas* spp. A 2.1-kb probe containing the 1.5-kb *phzO* gene and flanking regions was hybridized to total genomic DNA from 20 known phenazine-producing fluorescent pseudomonads to determine whether the gene is unique to producers of 2-hydroxyphenazines or if it also is conserved in other phenazine-producing strains. All seven strains of *P. aureofaciens* contained sequences that hybridized to the probe

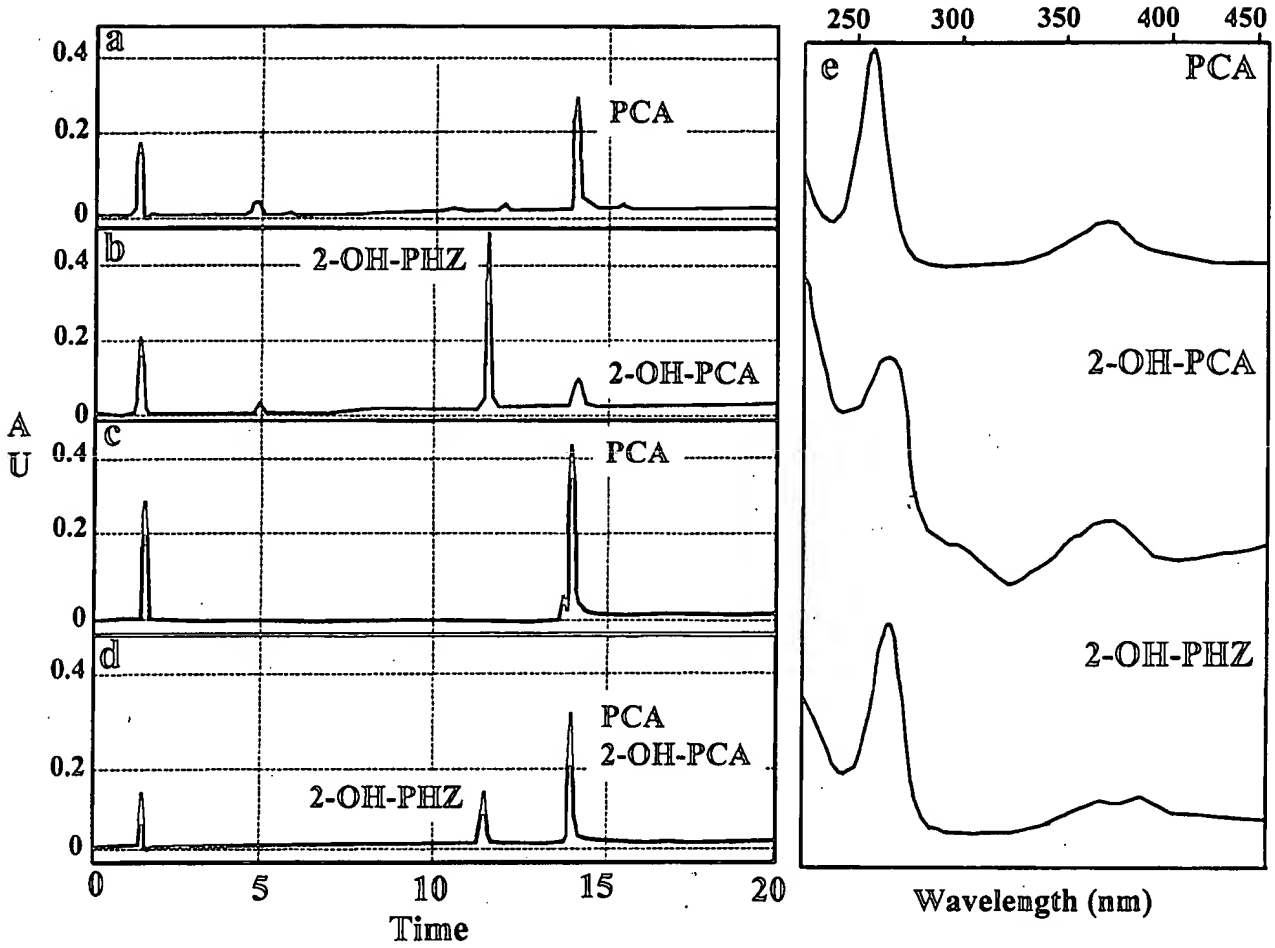

FIG. 2. HPLC analyses of phenazine compounds produced by *P. fluorescens* 2-79 harboring the pUCP26 vector (a), *P. fluorescens* 2-79 harboring pUCP2.9XP and containing *phzO* (b), *E. coli* JM109 harboring pUCP26 (c), *E. coli* JM109 harboring pUCP2.9XP and containing *phzO* (d), and peak identity of PCA and 2-OH-PHZ confirmed by spectral analysis (e). Retention times for PCA and 2-OH-PHZ are 14.1 and 11.4 min, respectively. Absorption maxima for PCA are 248 and 371 nm. Absorption maxima for 2-OH-PCA are 257 and 369 nm. Absorption maxima for 2-OH-PHZ are 257, 368, and 387 nm.

(Fig. 4), and each produced 2-OH-PCA and 2-OH-PHZ in addition to PCA, as determined by RP-HPLC. Two additional strains, *P. chlororaphis* 9446 and *P. aeruginosa* 25011, contained a faintly hybridizing band. However, no 2-hydroxylated phenazines were found in the extracts from cultures of these strains (data not shown), which previously were reported to produce chlororaphin and aeruginosins A and B, respectively (24, 54). No hybridization was detected between the *phzO* probe and DNA from *P. chlororaphis* strains ATCC 17411 and ATCC 17809, *P. aeruginosa* strains PA01, PAK-N1, PAK-NP1, PAK-NP2, ATCC 25007, and ATCC 25011, or the PCA-producing *P. fluorescens* strains 2-79, UQ 112, UN 4127, and UN 15 (Fig. 4), even after very heavy overexposure of the films (data not shown).

TABLE 3. Proteins displaying similarity to PhzO

Organism	Protein name	Enzyme	NCBI accession no.	Identity (%)[a]	Similarity (%)[a]	*Z* score[a]
Bacillus thermoleovorans A2	PheA	Phenol hydroxylase	AAC38324	28.1	37.9	42.1
Klebsiella pneumoniae	HpaA	4-hydroxyphenylacetate-3-hydroxylase	AAC37120	24.8	36.5	44.0
Escherichia coli ATCC 11105	HpaB	4-hydroxyphenylacetate hydroxylase	CAA82321	25.2	36.5	35.8
Ralstonia pickettii DTP0602	HadA	Chlorophenol-4-hydroxylase	BAA13105	26.7	39.9	64.4
Pseudomonas aeruginosa PAO1	PvcC	4-hydroxylphenylacetate hydroxylase	AAC21673	24.5	35.5	61.8
Burkholderia cepacia AC1100	TftD	Chlorophenol-4-monooxygenase	AAC23548	25.1	37.1	65.0
Photorhabdus luminescens	HpaB	4-hydroxylphenylacetate hydroxylase	AAC08739	24.0	35.9	37.0

[a] Identities, similarities, and *Z* scores were determined by the program BESTFIT from the Genetics Computer Group package.

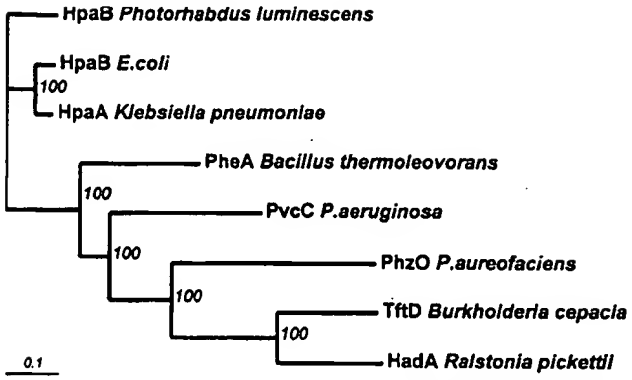

FIG. 3. Phylogenetic relationship between PhzO and various bacterial aromatic monooxygenases. The neighbor-joining tree with bootstrap support was constructed and visualized by using the CLUSTAL W and TreeView version 1.5.0 programs (31), respectively.

Fungal inhibition assays. Assays were conducted in vitro to determine if strains producing hydroxyphenazine compounds inhibited the hyphal growth of *G. graminis* var. *tritici* more than those producing only PCA. The radial growth of the fungus on plates in the presence of strain 30-84 was significantly less than that in the presence of the mutant 30-84mxO, which produced only PCA (18 versus 22 mm, $P \leq 0.05$). Similarly, *P. fluorescens* 2-79(pUCP2.9XP), transformed to hydroxyphenazine production, was more inhibitory than wild-type 2-79 (14 versus 17 mm, $P \leq 0.05$).

DISCUSSION

Results of the current study show clearly that *phzXYFABCD*, the core phenazine biosynthetic operon of strain 30-84, is responsible only for the synthesis of PCA (Fig. 5). When transformed with these genes, the sole phenazine product synthesized by *P. fluorescens* strains Q8r1-96 and M4-80R (which themselves do not produce phenazines) was PCA. A novel gene designated *phzO* was identified immediately downstream of the core biosynthetic operon of strain 30-84. Phenazine-nonproducing strains transformed with *phzXYFABCD* and *phzO*, or *P. fluorescens* 2-79 transformed with *phzO*, synthesized hydroxyphenazine compounds in addition to PCA, and *E. coli* expressing *phzO* rapidly converted exogenously supplied PCA to hydroxyphenazine products. Finally, a mutant of 30-84 inactivated in *phzO* produced only PCA. These results are consistent with those of an earlier study (27) suggesting that minor sequence differences between PhzF of strain 2-79 and PhzC of strain 30-84 are insufficient to account for the differences in the products synthesized by the two strains and implicating additional determinants of phenazine product specificity. This hypothesis is further supported by data reported by Chin-A-Woeng et al. (8), who recently described an aminotransferase gene designated *phzH* located downstream of the phenazine operon in *P. chlororaphis* PCL1391. PhzH was found to be responsible for the conversion of PCA to phenazine-1-carboxamide (chlororaphin), the green phenazine compound characteristic of *P. chlororaphis*. It thus appears that the presence of species-specific phenazine-modifying genes adjacent to the core biosynthetic locus may be a common feature among fluorescent *Pseudomonas* spp.

That PhzO belongs to a recently defined (14) family of two-component nonheme flavin-diffusible bacterial aromatic monooxygenases (TC-FDMs) is supported by results of both pairwise comparisons (high *Z* scores) and multiple sequence alignments (high bootstrap values). These enzymes are NAD(P)H-dependent flavoproteins that lack the defined GXGXXG FAD/NADH binding site typical of aromatic monooxygenases. Instead, they function in concert with a reductase component that uses NAD(P)H to generate a reduced flavin. The flavin then diffuses to the oxygenase, where it serves as a cosubstrate in the oxidation of aromatic compounds by molecular oxygen (14, 53). TC-FDMs hydroxylate aromatic substrates in either the *ortho* or the *para* position and include both dehalogenating (HadA from *Ralstonia pickettii*, PheA from *Bacillus thermoleovorans*, and TftD from *Burkholderia cepacia*) and nondehalogenating (HpaB from *E. coli*, HpaB from *Photorhabdus luminescens*, HpaA from *Klebsiella pneumoniae*, and PvcC from *P. aeruginosa*) enzymes (10, 14, 16, 23, 34, 42, 43). Many require the presence of an additional 19- to 21-kDa "coupling" subunit (16, 23, 34, 43) to provide reduced flavin, but at least for HpaB, the archetype of this family, this requirement can be satisfied with reduced flavin adenine dinucleotide provided exogenously or generated by an alternative flavin reductase (14, 53). This apparently is also the case for PhzO, since the cloned gene in either pUCP2.9XP or pGEM-PHZO (which contained very little flanking sequence from *P. aureofaciens* 30-84) was sufficient to catalyze the conversion of PCA to 2-hydroxyphenazines in *E. coli*. Whereas the genes encoding the oxidase and reductase components of most known TC-FDM enzymes are situated near one other on the chromosome (14), we found no detectable similarity with known flavin reductases in the 1.3-kb DNA segment downstream of *phzO*. Although a functionally "dedicated" reductase may be encoded elsewhere in the genome of strain 30-84, such an enzyme clearly is not required for phenazine 2-hydroxylation in *E. coli*. The apparent absence of a linked reductase gene and the relatively low level of overall homology between PhzO and other members of the TC-FDM family distinguish this phenazine-modifying enzyme from other oxygenases.

Earlier, Flood et al. (13) in a study with deuterated precursors revealed that hydroxylated phenazines are synthesized in *P. aureofaciens* through the formation of a hypothetical arene intermediate in the following order: PCA→2-OH-PCA→2-OH-PHZ (13). The authors also concluded that the hydroxylation of PCA occurred inefficiently, since PCA was more abundant in the extracts than were the hydroxylated derivatives. Based on the results of our study, we speculate that 2-hydroxylation of PCA is carried out in *P. aureofaciens* by a nonheme, flavin-diffusible monooxygenase, PhzO, which adds a hydroxyl group to PCA at the *ortho* position relative to the carboxyl group, resulting in the synthesis of 2-OH-PCA (Fig. 5). The reaction presumably also requires a yet-unidentified, highly active reductase, NAD(P)H, flavin, and O_2. As speculated previously (13), the subsequent decarboxylation of 2-OH-PCA to 2-OH-PHZ occurs spontaneously in the absence of enzymes. Up to 74% of 2-OH-PCA in phosphate buffer at pH 7 was converted to 2-OH-PHZ after 18 h, whereas lesser amounts (33 and 62%) were converted in buffers at pH values

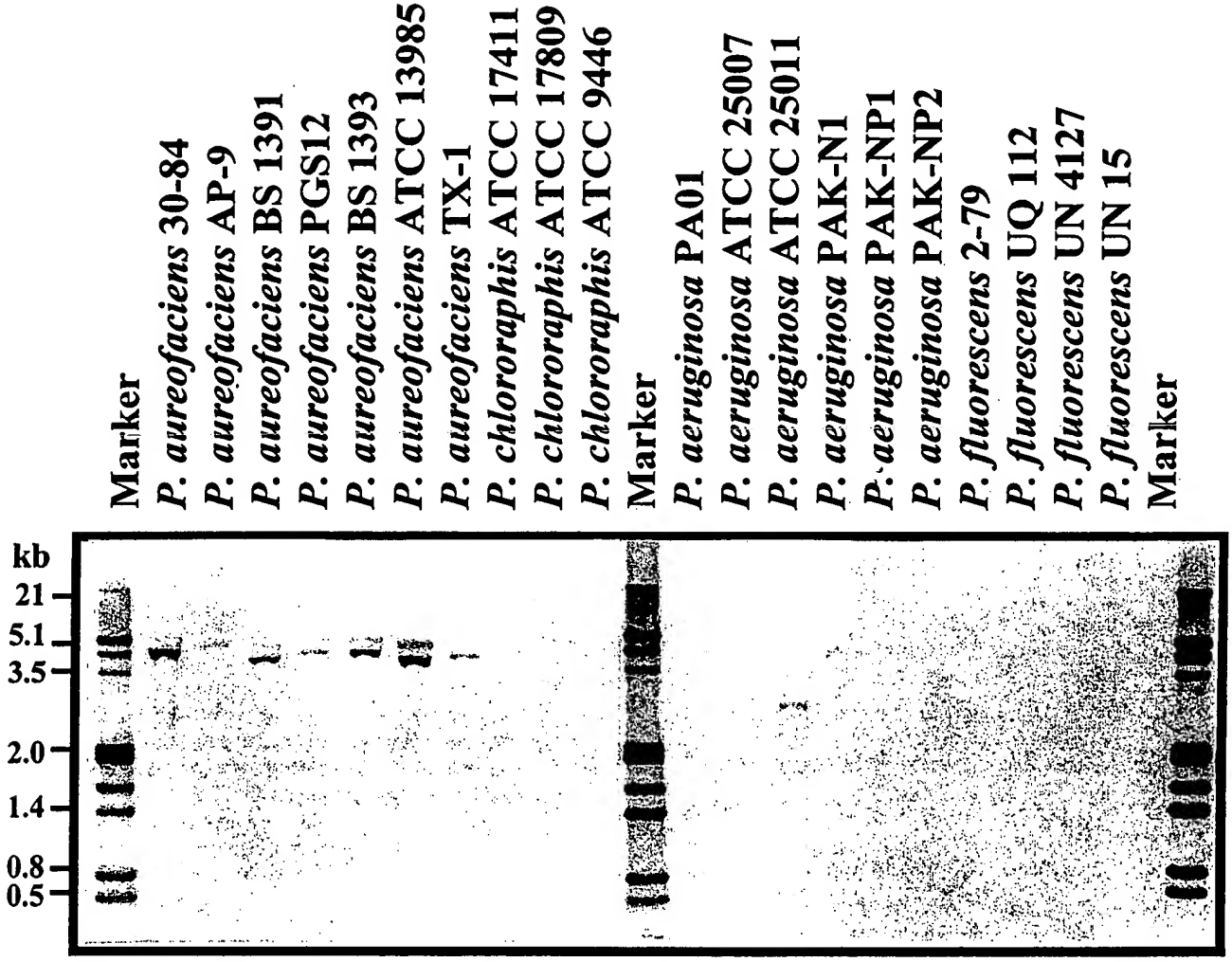

FIG. 4. Southern hybridization of *phzO* probe to total genomic DNA from 20 phenazine-producing *Pseudomonas* strains. Total DNA samples were digested with endonucleases *Pst*I and *Eco*RI.

of 6 and 8, respectively (G. Phillips and L. S. Thomashow, unpublished data).

We screened a collection of phenazine-producing *Pseudomonas* spp. for the presence of *phzO* by Southern hybridization (Fig. 4). Our results indicate that this gene is found almost exclusively in isolates of *P. aureofaciens*. The only two non-*P. aureofaciens* strains that hybridized with the *phzO* probe were *P. chlororaphis* 9446 and *P. aeruginosa* 25011 (Fig. 4). However, it is possible that these strains do not have the *phzO* homologue, since in both cases the hybridization signal was very weak, the size of the hybridizing fragment was different from that in *P. aureofaciens* strains, and no hydroxylated phenazines

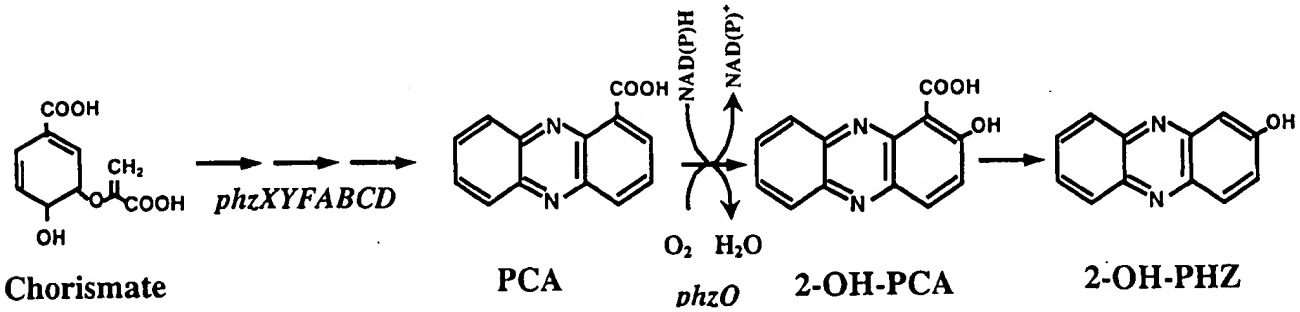

FIG. 5. Proposed mechanism for the production of 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine in *P. aureofaciens* 30-84.

were detected in the culture extracts. Based on these findings, we speculate that *phzO* (and probably *phzH*) is a species-specific gene in fluorescent *Pseudomonas* spp. Moreover, the fact that all the tested strains possess a well-conserved core phenazine locus (D. M. Mavrodi and L. S. Thomashow, unpublished observation) may indicate that the acquisition of phenazine-modifying genes by phenazine-producing pseudomonads is a fairly recent event.

Interest in strains of *P. aureofaciens* frequently has centered on their ability to suppress soilborne plant pathogens (4, 32, 40, 45). We used derivatives of strain 30-84 mutated in *phzO* and strain 2-79 transformed with *phzO* to evaluate the importance of hydroxylated phenazines in biological control activity against *G. graminis* var. *tritici* in vitro. For both strains, the ability to produce hydroxyphenazine compounds was correlated with greater antifungal activity than was production of PCA alone. These results are consistent with the findings of Smirnov and Kiprianova (40), who compared the inhibitory effects of PCA, 2-OH-PCA, and 2-OH-PHZ against a variety of bacterial and fungal animal and plant pathogens and found that in all cases the 2-hydroxyphenazines exhibited stronger bacteriostatic and fungistatic activity. We have recently demonstrated that the introduction of the core biosynthetic genes in other biocontrol microorganisms resulted in increased suppression of certain phytopathogenic fungi (46a; Huang et al., unpublished data). The *phzO* gene from *P. aureofaciens* 30-84 is an attractive target for such genetic manipulations because of the wide antimicrobial and antifungal activity of 2-hydroxyphenazines, which, on the other hand, exhibit little or no toxicity to fish, insects, or mammals (29, 47).

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A Seven-Gene Locus for Synthesis of Phenazine-1-Carboxylic Acid by *Pseudomonas fluorescens* 2-79

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***Pseudomonas fluorescens* 2-79 produces the broad-spectrum antibiotic phenazine-1-carboxylic acid (PCA), which is active against a variety of fungal root pathogens. In this study, seven genes designated *phzABCDEFG* that are sufficient for synthesis of PCA were localized within a 6.8-kb *Bgl*II-*Xba*I fragment from the phenazine biosynthesis locus of strain 2-79. Polypeptides corresponding to all *phz* genes were identified by analysis of recombinant plasmids in a T7 promoter/polymerase expression system. Products of the *phzC*, *phzD*, and *phzE* genes have similarities to enzymes of shikimic acid and chorismic acid metabolism and, together with PhzF, are absolutely necessary for PCA production. PhzG is similar to pyridoxamine-5′-phosphate oxidases and probably is a source of cofactor for the PCA-synthesizing enzyme(s). Products of the *phzA* and *phzB* genes are highly homologous to each other and may be involved in stabilization of a putative PCA-synthesizing multienzyme complex. Two new genes, *phzX* and *phzY*, that are homologous to *phzA* and *phzB*, respectively, were cloned and sequenced from *P. aureofaciens* 30-84, which produces PCA, 2-hydroxyphenazine-1-carboxylic acid, and 2-hydroxyphenazine. Based on functional analysis of the *phz* genes from strains 2-79 and 30-84, we postulate that different species of fluorescent pseudomonads have similar genetic systems that confer the ability to synthesize PCA.**

Certain members of the genus *Pseudomonas* produce diverse low-molecular-weight ("secondary") metabolites including nitrogen-containing heterocyclic pigments known as phenazine compounds (5, 19). Phenazines are synthesized by a limited number of bacterial genera including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, and *Streptomyces* (38). Almost all phenazines exhibit broad-spectrum activity against various species of bacteria and fungi (32). This activity is connected with the ability of phenazine compounds to undergo oxidation-reduction transformations and thus cause the accumulation of toxic superoxide radicals in the target cells (15). Some phenazine compounds can act as bacterial virulence factors. For example, pyocyanin, produced by the opportunistic pathogen *Pseudomonas aeruginosa* during cystic fibrosis, has been shown to inhibit the ciliary function of respiratory epithelial cells (40). Phenazine antibiotics produced by the biocontrol strains *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 are major factors in the ability of these strains to inhibit the growth of fungal root pathogens. Moreover, studies involving phenazine-deficient mutants have clearly demonstrated that antibiotic production in natural habitats plays an important role in the ecological competence and long-term survival of these strains in the environment (21). Over 50 naturally occurring phenazine compounds have been described, and certain bacterial producers are able to synthesize mixtures of as many as 10 different phenazine derivatives at one time (32, 38). Growth conditions also may influence the number and types of phenazines synthesized by an individual strain (38).

Early studies with radiolabeled precursors revealed tight links in several microorganisms between biosynthesis of phenazine compounds and the shikimic acid pathway (38). Phenazine-1,6-dicarboxylic acid is believed to be the first phenazine formed and to be the one from which others are derived (19). It also was proposed that the phenazine nucleus is formed by the symmetrical condensation of two molecules of chorismic acid and that enzymes involved in this conversion must have many features in common with anthranilate synthases (17). Despite intensive biochemical studies, the biosynthetic intermediates have not been identified and little is known about the genetics of phenazine synthesis (38). To date, the best-studied phenazine genes are those cloned from *P. aureofaciens* 30-84 (26, 27). The products of the *phz* structural genes from strain 30-84 are similar to enzymes from the shikimic acid and tryptophan biosynthetic pathways, confirming predictions from earlier biochemical analyses. Two other genes, *phzI* and *phzR*, encode parts of a quorum-sensing circuit that regulates phenazine production in *P. aureofaciens* 30-84 in a cell density-dependent manner (26).

In this paper, we present organizational and functional analyses of the complete genetic locus for phenazine-1-carboxylic acid biosynthesis from *P. fluorescens* 2-79. Two new genes from the homologous locus of *P. aureofaciens* 30-84 also are described, and the structure and function of the biosynthetic gene clusters from the two strains are compared. Results of this study suggest that the mechanism of phenazine biosynthesis is highly conserved among fluorescent *Pseudomonas* species.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. A spontaneous rifampin-resistant derivative of *P. fluorescens* 2-79 was used in all studies. *P. fluorescens* 2-79 and *P. aureofaciens*

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
P. fluorescens 2-79	Phz$^+$ wild type	36
P. fluorescens 2-79RN$_{10}$	Phz$^+$ Rif^r Nal^r	36
P. fluorescens 2-79MXC	Phz$^-$ Rif^r *phzC*::Kan^r	This study
P. fluorescens 2-79MXD	Phz$^-$ Rif^r *phzD*::Kan^r	This study
P. fluorescens 2-79MXE	Phz$^-$ Rif^r *phzE*::Kan^r	This study
P. fluorescens 2-79MXG	Phz$^+$ Rif^r *phzG*::Kan^r	This study
P. fluorescens 2-79.8A	Phz$^-$ Rif^r *phzE*::Tn*5*	37
P. aureofaciens 30-84	Phz$^+$ wild type	25
E. coli JM109	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15/recA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	Promega
E. coli HB101	Δ(*gpt-proA*)*62 leuB6 thi-1 lacY1 hsdS*$_B$*20 recA rpsL20 ara-14 galK2 xyl-5 mtl-1 supE44 mcrB*$_B$	1
E. coli S17-1	*thi pro hsdR hsdM recA rpsL* RP4-2 (Tet^r::Mu) (Kan^r::Tn*7*)	31
E. coli BL21	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm*	35
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (λc*Its*857 *ind1 Sam7nin5 lacUV5*-T7gene1)	35
E. coli BL21(DE3)/pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (λc*Its*857 ind1 Sam7nin5 lacUV5-T7gene1)pLysS	23
E. coli C2110	K-12 *gyrA polA1 his rha*	34
Plasmids		
pBluescript II KS(SK)	ColE1 f1(+/−) *bla*	Stratagene
pT7-5, pT7-6	T7 promoter ColE1 *bla*	1
pET-3a	T7 promoter ColE1 *bla*	35
pGP1-2	Source of T7 RNA polymerase p15A	1
pUC4K	Source of Kan^r cassette ColE1 *bla*	Pharmacia
pJQ200SK	p15A *aacC1* Mob$^+$ *sacB*	29
pHoHo	Tn*3* delivery vector *lacIZYA*$^+$ *tnpA bla*	34
pSShe	Helper plasmid for Tn*3* mutagenesis; *tnpA cat*	34
pRK2013	Helper plasmid; ColE1 Kan^r Mob$^+$ Tra$^+$	9
pPHZ108A	pLAFR3 containing *P. fluorescens* 2-79 genomic DNA, Phz$^+$	37
pT7-5FABCD	pT7-5 containing 5.7-kb *Eco*RI-*Hin*dIII fragment with *phzFABCD* genes from *P. aureofaciens* 30-84	This study
pT7-5X-D	pT7-5 containing 6.9-kb *Pst*I-*Hin*dIII fragment with *phzXYFABCD* genes from *P. aureofaciens* 30-84	This study
pT7-6A-G	pT7-6 containing 6.9-kb *Bgl*II-*Xba*I fragment from pPHZ108A with *phzABCDEFG* genes	This study
pT7-6ABCD	pT7-6 containing 4.5-kb *Bgl*II-*Pst*I fragment from pPHZ108A with *phzABCD* genes	This study
pT7-6AB	pT7-6 containing 1.3-kb *Bgl*II-*Eco*RV fragment from pPHZ108A with *phzAB* genes	This study
pT7-6B	pT7-6 containing 1.4-kb *Xho*I-*Eco*RV fragment from pPHZ108A with *phzB* gene	This study
pT7-5CD	pT7-5 containing 2.0-kb *Eco*RI-*Kpn*I fragment from pPHZ108A with *phzCD* genes	This study
pT7-5CDE	pT7-5 containing 4.3-kb *Eco*RI-*Bgl*II fragment from pPHZ108A with *phzCDE* genes	This study
pT7-6CDEFG	pT7-6 containing 5.8-kb *Eco*RI-*Xba*I fragment from pPHZ108A with *phzCDEFG* genes	This study
pT7-5G	pT7-5 containing 1.5-kb *Bgl*II-*Xba*I fragment from pPHZ108A with *phzG* gene	This study
pET-3XY	pET-3a containing *phzX* and *phzY* genes	This study

[a] *bla*, β-lactamase; *aaC1*, gentamicin acetyltransferase-3-1; *cat*, chloramphenicol acetyltransferase; Kan^r, kanamycin resistance; Rif^r, rifampin resistance; Nal^r, nalidixic acid resistance.

30-84 were grown at 28°C in Luria-Bertani (LB) broth (1). *E. coli* strains were grown at 28 or 37°C in LB or M9 minimal medium (1). Antibiotic supplements were used at the following concentrations: ampicillin, 80 μg/ml; carbenicillin, 80 μg/ml; rifampin, 75 μg/ml; kanamycin, 30 or 150 μg/ml; tetracycline, 12.5 μg/ml; gentamicin, 10 μg/ml.

Transposon Tn*3-lacZ* mutagenesis. Tn*3-lacZ* insertions were made by using the transposon system described by Stachel et al. (34). The target cosmid, pPHZ108A, was introduced into *E. coli* HB101(pHoHo1, pSShe). To select pPHZ108A::Tn*3-lacZ* derivatives, the strain harboring pPHZ108A, pHoHo1, and pSShe was mated with recipient strain *E. coli* C2110, using *E. coli* HB101(pRK2013) as a helper. The site and orientation of insertions into pPHZ108A were analyzed by restriction mapping. The plasmids were then introduced into the phenazine-1-carboxylic acid (PCA)-nonproducing Tn*5* mutant *P. fluorescens* 2-79.8A by triparental matings to test the effect of each Tn*3-lacZ* insertion on PCA production. Expression in transconjugants of the *lacZ* reporter gene, an indicator of transcriptional activity, was detected on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal).

DNA manipulations. Standard methods were used for DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (1). Total DNA from *P. fluorescens* 2-79RN$_{10}$ was isolated and purified by a cetyltrimethylammonium bromide miniprep procedure (1). For Southern blotting and hybridization, total DNA samples were digested with restriction endonucleases, separated by electrophoresis in a 0.8% agarose gel, transferred onto a BrightStar-Plus nylon membrane (Ambion, Inc., Austin, Tex.), and hybridized with a specific DNA probe labeled with the DECAprime-Biotin random priming kit (Ambion, Inc.). DNA-DNA hybrids were detected with the BrightStar nonisotopic detection kit (Ambion, Inc.) as specified by the manufacturer.

Cloning of *phzX* and *phzY* genes from *P. aureofaciens* 30-84. A DNA fragment containing *phzX* and *phzY* genes was amplified with *P. aureofaciens* 30-84 genomic DNA as a template and with oligonucleotide primers 30-84/1 (5′-CAG TTCATCCGGCGGGCTGCAG-3′) and 30-84/2 (5′-CCCGTTTCAGTAAGTC TTCCATGATGCG-3′). Target DNA was amplified with Vent DNA polymerase (New England Biolabs, Beverly, Mass.) and the following cycling program: 94°C for 1 min, 64°C for 45 s, and 72°C for 1 min (30 cycles). The 1.2-kb PCR product was cloned into the *Sma*I site of pBluescript II KS, and the resulting plasmid was used to determine the nucleotide sequence.

DNA sequencing and analysis. DNA was sequenced by the dideoxy chain termination method with Sequenase 2.0 (Amersham International, Little Chalfont, United Kingdom). Exonuclease III deletion derivatives were constructed with enzymes supplied by Amersham, as specified by the manufacturer. Sequence data were compiled and analyzed with the GCG package (13). DNA sequences were compiled with GELASSEMBLE, and open reading frames (ORFs) and codon usage were analyzed with MAP and FRAMES. A database search for similar protein sequences was carried out with the BLAST and FASTA network servers at the National Center for Biotechnology Information and the European Molecular Biology Laboratory, respectively. The probable domain homology search was performed with the PROSITE (European Molecular Biology Laboratory, Heidelberg, Germany) and SBASE (International Cen-

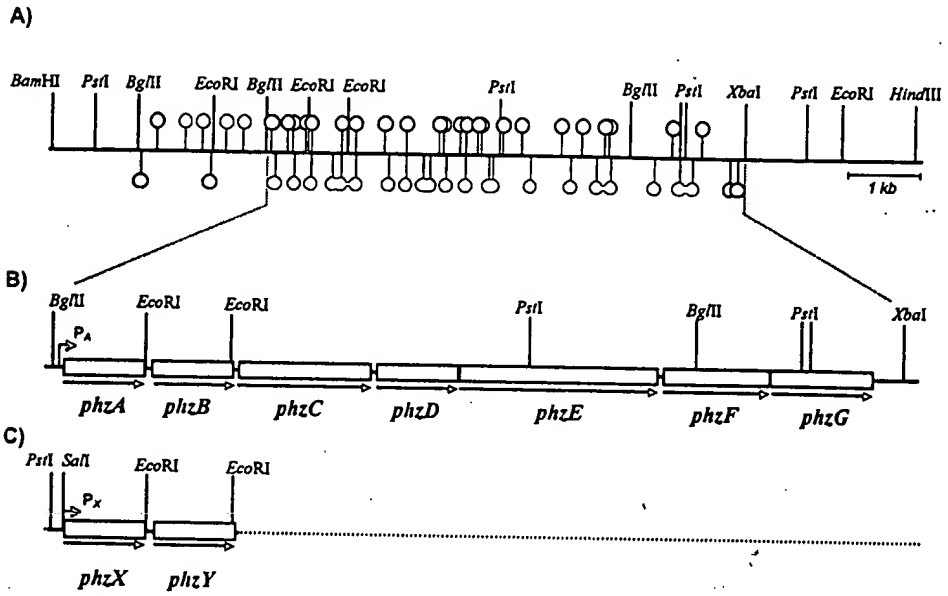

FIG. 1. Physical maps of cosmid clone pPHZ108 (A), the DNA region from *P. fluorescens* 2-79 encoding genes involved in the production of PCA (B), and a portion of the homologous genetic locus from *P. aureofaciens* 30-84 (C). Open boxes indicate genes encoding phenazine biosynthesis enzymes. The direction of the gene transcription is shown by an arrow. The symbols P_A and P_X represent the position and orientation of corresponding promoters. Insertions of Tn*3-lacZ* that interfered with phenazine production are marked on the map of pPHZ108A as ↑ (Lac$^+$ phenotype) and ↑ (Lac$^-$ phenotype).

ter for Genetic Engineering and Biotechnology, Trieste, Italy) computer servers (3, 12). Pairwise alignments were obtained with the GCG GAP program (gap weight = 4).

Analyses of polypeptide gene products. The *P. fluorescens* 2-79 structural *phz* genes were expressed under the control of the T7 promoter in plasmid vectors pT7-5 and pT7-6 in *E. coli* BL21 with pGP1-2 as a source of T7 RNA polymerase and with [^{35}S]methionine for selective labeling of target proteins (1). Labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

For expression studies of *phzX* and *phzY* from *P. aureofaciens* 30-84, the genes were cloned into the *NdeI-Bam*HI sites of pET-3a vector (35) after amplification with the oligonucleotide primers phz*XNde*I (5′-TTTTTTCATATGCCTGCTTC GCTTTC-3′) and phz*YBam*HI (5′-TTTGGATCCTTAAGTTGGAATGCCTT CG-3′) and subsequent cleavage of the PCR product with the corresponding restriction endonucleases. Expression of the *phzX* and *phzY* genes was carried out in *E. coli* BL21(DE3)/pLysS (23). Total cellular protein was separated by SDS-PAGE and stained with Coomassie brilliant blue as described elsewhere (7).

Gene replacement experiments in *P. fluorescens* 2-79. A 1.4-kb Km^r cassette (Pharmacia Biotech, Inc., Uppsala, Sweden) containing the aminoglycoside 3′-phosphotransferase gene was used in gene replacement experiments. Plasmids containing individual *phz* genes that were insertionally inactivated by the antibiotic resistance gene were constructed. Target DNA fragments were then subcloned into pJQ200SK, a gene replacement vector harboring the *sacB* gene as a counterselectable marker (29). The resulting plasmids were transferred into *P. fluorescens* 2-79 via biparental matings with *E. coli* S17-1, followed by selection for the plasmid resistance marker. Subsequent selection was performed on LB agar containing 5% sucrose and kanamycin. Introduced mutations were verified at the DNA level by PCR screening and Southern hybridization.

Extraction and detection of phenazine compounds. Prior to extraction of phenazine compounds, *Pseudomonas* strains were cultivated in LB medium for 3 days at 28°C. *E. coli* BL21(DE3) clones harboring *phz* genes under control of the T7 promoter were grown in LB medium at 28°C without addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The "leaky" *lac* promoter of the λDE3 lysogen maintained high enough levels of T7 RNA polymerase in the cell to enable the production of detectable amounts of phenazines.

Phenazine compounds were extracted from 3-day-old cultures of *E. coli* and *Pseudomonas* strains with ethyl acetate by the method of Bonsall et al. (4). Filtered crude extracts were subjected to C_{18} reverse-phase high-performance liquid chromatography (HPLC) (Waters, Symmetry C_{18}; 5-μm particles of packing material; 3.0 by 150 mm) with a 30-μl injection volume. The Waters HPLC Integrity System consisted of an Alliance 2690 separation module, a 996 photodiode array detector, and a Thermobeam mass spectrometry detector. Solvent conditions included a flow rate of 350 μl/min with a 2-min initial condition of 10% acetonitrile–2% acetic acid followed by a 20-min linear gradient to 100% acetonitrile–2% acetic acid. HPLC gradient profiles were monitored at the spectral peak maxima (247.6 and 368.2 nm) that are characteristic of PCA in the designated solvent system. Mass spectrometry conditions included an ion source temperature of 220°C, an expansion region temperature of 80°C, a nebulizer temperature of 84°C, and a helium flow at 15 lb/in^2.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequence data for *phz* genes from *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 are L48616 and AF007801, respectively.

RESULTS

Mutagenesis of *P. fluorescens* 2-79 with Tn*3-lacZ*. The phenazine biosynthetic locus from *P. fluorescens* 2-79 was cloned previously as a 12-kb *Hin*dIII-*Bam*HI DNA fragment within plasmid pPHZ108A and characterized by restriction mapping (37). Mutagenesis of pPHZ108A with Tn*3-lacZ*, which can generate fusions in which expression of the *lacZ* gene is regulated by the promoter of the gene bearing the insertion, yielded 57 unique transposon insertions that interfered with phenazine production. These insertions identified two adjacent, divergently transcribed units of approximately 6 and 0.75 kb that were strongly and weakly expressed, respectively, under conditions favorable to the production of PCA (data not shown). Most of the large transcriptional unit was contained within a 5.4-kb *Bgl*II fragment, with the remainder localized to the adjacent 2.0-kb *Bgl*II-*Xba*I DNA fragment (Fig. 1). Consistent with results from Tn*3-lacZ* mutagenesis, the *Bgl*II fragment alone did not enable PCA production in *P. fluorescens* 2-79.8A, suggesting a requirement for additional downstream sequences.

DNA sequence analysis. The overlapping 5.7-kb *Eco*RI-*Xba*I and 5.4-kb *Bgl*II fragments and the adjacent 1.8-kb *Bgl*II fragment were subcloned into pBluescript II KS and SK cloning vectors and used to determine the nucleotide sequence of an 8,505-bp DNA segment from pPHZ108A. Computer analysis of the DNA sequence within the large transcriptional unit revealed seven ORFs with high coding probability, designated *phzABCDEFG*. Each of these genes is preceded by a well-conserved ribosome binding site. In the *phzD-phzE* and *phzF-*

TABLE 2. ORFs in the *phz* loci of *P. fluorescens* and *P. aureofaciens*

Gene	Protein length (residues)	Protein mol mass (kDa)	Similarity to:
phzA	163	18.7	PhzB from *P. fluorescens* 2-79; PhzX and PhzY from *P. aureofaciens* 30-84
phzB	162	18.8	PhzA from *P. fluorescens* 2-79; PhzX and PhzY from *P. aureofaciens* 30-84
phzC	400	44.0	PhzF from *P. aureofaciens* 30-84; plant phospho-2-dehydro-3-deoxyheptonate aldolases (DAHP synthases)
phzD	207	23.0	PhzA from *P. aureofaciens* 30-84; bacterial isochorismatases
phzE	637	69.9	PhzB from *P. aureofaciens* 30-84; bacterial class I glutamine amidotransferases
phzF	278	30.0	PhzC from *P. aureofaciens* 30-84; hypothetical proteins ORF o276#3 from *E. coli* and ORF slr1019 from *Synechocystis* sp.
phzG	222	24.9	PhzD from *P. aureofaciens* 30-84; bacterial pyridoxamine-5′-phosphate oxidases
phzX	166	19.2	PhzY from *P. aureofaciens* 30-84; PhzA and PhzB from *P. fluorescens* 2-79
phzY	163	18.8	PhzX from *P. aureofaciens* 30-84; PhzA and PhzB from *P. fluorescens* 2-79

phzG pairs, the stop (UGA) and start (AUG) codons of the adjacent genes overlap, possibly reflecting their translational coupling. Two additional genes homologous to *phzI* and *phzR* also were identified upstream of the *phzABCDEFG* cluster (20).

Homology searches of deduced amino acid sequences against the Swiss-Prot, GenPept, and PIR protein databases identified a number of proteins with significant similarities to the predicted protein products of the *phzABCDEFG* genes (Table 2). Apart from the results listed and discussed below, products of the *P. fluorescens* 2-79 *phzCDEFG* genes also were highly homologous (above 90% identity and similarity) to the products of the *phzFABCD* genes, respectively, from *P. aureofaciens* 30-84 (27).

No similarities for the PhzA and PhzB proteins, or for the homologous proteins PhzX and PhzY from *P. aureofaciens* 30-84, were identified in database searches. On the other hand, all four polypeptides were remarkably homologous, with similarity increasing toward the C-terminal part of the proteins (62% overall identity and 24.7% similarity) (Fig. 2).

The 400-amino-acid PhzC protein showed 39.1% identity and 46.1% similarity to the AroG 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase from *Lycopersicon esculentum* chloroplasts, as well as to other plant DAHP synthases (Swiss-Prot accession no. P37216, P21357, and P27608). DAHP synthase is the first enzyme of the shikimate pathway and catalyzes the condensation of phosphoenolpyruvate and erythrose-4-phosphate. In contrast to bacterial DAHP synthase isoenzymes, which are feedback inhibited by one of the aromatic amino acids, plant DAHP synthases are not inhibited by aromatic acids but are activated by tryptophan (10, 28).

The *phzD* gene encodes a small protein of 207 amino acid residues that is homologous (46.9% identity and 59.9% similarity) to the 285-amino-acid isochorismatase EntB from *E. coli* (11). Isochorismatase is an enzyme from the biosynthetic pathway of enterobactin—an iron-chelating product derived from chorismic acid and involved in the transport of iron from the bacterial environment into the cell cytoplasm.

The results of BLAST search analyses revealed that PhzE was similar to a large group of enzymes including anthranilate synthase from *Streptomyces venezuelae* (45.9% identity and 33.2% similarity), other bacterial anthranilate synthases, *p*-aminobenzoate synthases, and menaquinone-specific isochorismate synthases. According to the PROSITE database search results, the C-terminal part of PhzE (amino acid residues 438 to 637) resembles a class I glutamine amidotransferase (GATase) containing a well-conserved putative active site PFLAVCLSHQVL (letters in boldface type indicate highly conserved residues, and the essential cysteine is underlined). GATases are a large group of biosynthetic enzymes able to catalyze the removal and transfer of the ammonia group from glutamine to various substrates, forming a new carbon-nitrogen bond. The GATase domain exists either as a separate polypeptide subunit or as part of a larger polypeptide fused in different ways to a synthase domain (28).

The product of the *phzF* gene shows weak similarity to a number of hypothetical proteins of unknown function listed in the databases. The latter include ORF o276#3 from *Escherichia coli* (GenBank accession no. D90786), ORF slr1019 from *Synechocystis* sp. (GenBank accession no. D90904), and the hypothetical 32.6-kDa protein YHI9 from *Saccharomyces cerevisiae* (Swiss-Prot accession no. P38765).

PhzG shows 29.3% identity and 38.1% similarity to the PdxH pyridoxamine-5′-phosphate oxidase from *E. coli* and to the similar protein from *H. influenzae* (Swiss-Prot accession no.

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PhzA  M---PGSLSSGGFNDHLELRRKNRATVDQYMRTNGEDRLRRHELFTPDGS  47
PhzX  MLPMPASLSPSGFNDHLELRQKNRATVEQYMRTNGKDRLRRHELFTQDGS  50
PhzB  M-PDSTVQQPIT-DDT-ELRRKNRATVEQYMRTKGQDRLRRHELFTEDGS  47
PhzY  M-SNSAAQQLTA-NDTTELRRKNRATVEQYMRTKGQDRLRRHELFTEDGT  48
      *  ..    . .*  ***.******.*****.*.********** **.

PhzA  GGSWNTETGEPLVFKGHAKLAALGVWLHQCFPDWQWHNVRVFETDNPNHF  97
PhzX  GGSWNTETGKPLVFKGHTKLAALGVWLEKCFPDWQWHNVRVFETDNPNHF 100
PhzB  GGLWTTDTGAPIVISGKAKLAEHAVWSLKCFPDWEWYNVKVFETDDPNHI  97
PhzY  GGLWTTDTGAPIVISGKAKLAEHAVWSLKCFPDWEWYNVKVFETDDPNHI  98
      ** *.*.** *.*..*..***. .**  .*****.*.**.*****.***.

PhzA  WVESDGRGTTRVPGYPEGYCENHYIHSFELDNGKITQNREFMNPFEQLRA 147
PhzX  WVESERRGKTLVPGYPEGYCENHYIHSFELDDGKITQSREFMNPFEQLRA 150
PhzB  WVECDGHGKILFPGYPEGYYENHFLHSFELQDGKVKRNREFMNVFQQLRA 147
PhzY  WVECDGHGKILFPGYPEGYYENHFLHSFELEDGKVKRNREFMNVFQQLRA 148
      ***.. .*..  *******.***..*****..**....***** *.****

PhzA  LGIPVPKIKREGIPAS 163
PhzX  LGIPVPRIKREGIPAS 166
PhzB  LGIPVPHIKREGIPA- 162
PhzY  LGIPVPQIKREGIPT- 163
      ******.*******.
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FIG. 2. Alignment of the deduced amino acid sequences of the PhzA (*P. fluorescens* 2-79), PhzX (*P. aureofaciens* 30-84), PhzB (*P. fluorescens* 2-79), and PhzY (*P. aureofaciens* 30-84) proteins. Identical (*) and similar (·) amino acid sequences are indicated. Dashes represent gaps inserted in the sequences to improve the alignment.

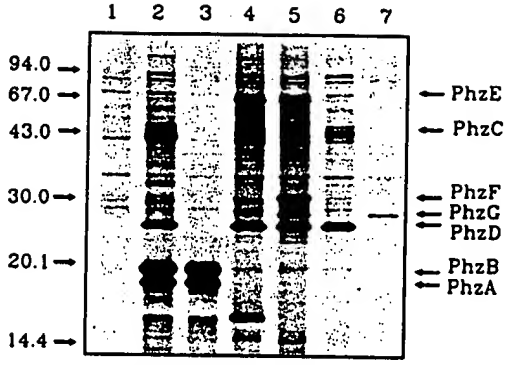

FIG. 3. Autoradiograph of polypeptides labeled with [^{35}S]methionine and resolved by electrophoresis on an SDS–12% polyacrylamide gel. Samples were prepared from *E. coli* BL21 containing pGP1-2 plus the indicated plasmids. The location of DNA fragments inserted in pT7-5 and pT7-6 vectors for gene expression is shown in Fig. 4. Lanes: 1, pT7-6; 2, pT7-6ABCD; 3, pT7-6AB; 4, pT7-5CDE; 5, pT7-6CDEFG; 6, pT7-5CD; 7, pT7-5G. An intense 16-kDa band in lane 4 is a product of a truncated *phzF* gene, and an intense protein band almost overlapping with PhzC in lane 2 is a product of a truncated *phzE* gene. Positions of molecular size markers are given on the left in kilodaltons.

P28225 and P44909). These enzymes are involved in de novo synthesis of pyridoxine (vitamin B_6) and pyridoxal phosphate in bacterial cells, and they convert pyridoxamine-5′-phosphate into pyridoxal-5′-phosphate (18). Moreover, the results of a PROSITE database search revealed that part of PhzG (amino acid residues 192 to 205) possesses an amino acid sequence motif, **LEFWGNGQERLHER** (letters in boldface indicate highly conserved residues), characteristic of all bacterial pyridoxamine-5′-phosphate oxidases studied to date.

Identification of proteins encoded by *phzABCDEFG*, *phzX*, and *phzY*. Using a pair of pT7-5 and pT7-6 transcriptional vectors and the two-plasmid expression system of Tabor, we identified products for all of the *phzABCDEFG* genes (Fig. 3). The rather high level of expression of all seven genes reflects efficient utilization of the *Pseudomonas* translational signals by the *E. coli* protein-synthesizing machinery.

Despite the very similar sizes of the PhzA and PhzB proteins (18.7 and 18.8 kDa, respectively), they could be resolved by SDS-PAGE (Fig. 3, lanes 2 and 3). The single PhzB polypeptide also was identified by expression from pT7-6B (data not shown). Expression of the *phzC* gene was confirmed with several different plasmids, and in most cases a faint protein band of about 44 kDa was detected (lane 6). The unusual appearance of this protein in the gels probably is a result of partial proteolysis of the PhzC polypeptide in *E. coli* cells. The *phzD*, *phzE*, *phzF*, and *phzG* genes were well expressed, and the sizes of their products estimated by SDS-PAGE were in agreement with those predicted from nucleotide sequences (lanes 2, 4, 5, and 6). The comparatively poor expression of *phzE* (data not shown) and *phzG* (lane 7) genes alone is consistent with their likely translational coupling with the *phzD* and *phzF* genes, respectively. According to results from Northern blotting experiments, the *phzABCDEFG* genes form a distinct operon in *P. fluorescens* 2-79 and are transcribed as a single mRNA (20).

The *phzX* and *phzY* genes from *P. aureofaciens* 30-84 were expressed in *E. coli* BL21(DE3)/pLysS with a pET3a translational vector (data not shown). The level of expression was very high, and the PhzX and PhzY proteins (19.2 and 18.8 kDa, respectively) were easily detected after SDS-PAGE by a Coomassie blue staining procedure.

Functional analysis of the *phz* genes. To determine which genes in the phenazine biosynthetic cluster are essential for the production of PCA, the Kan^r cassette was inserted into *phzC*, *phzD*, *phzE*, and *phzG* and each disrupted gene was introduced into the genome of strain 2-79 by homologous recombination. For unknown reasons, it was not possible to recover *phzA*, *phzB*, or *phzF* recombinants. Insertions of the Kan^r cassette in *phzC*, *phzD*, and *phzE* interfered with phenazine production, and the corresponding mutant strains were completely deficient in PCA biosynthesis (Fig. 4A). *P. fluorescens* 2-79 MXG, bearing a Kan^r insertion within *phzG*, was able to produce PCA, although the yield was only about 1.3% of that of wild-type strain 2-79.

Combinations of genes from the 2-79 phenazine gene cluster also were cloned under control of a T7 promoter and tested in *E. coli* for the ability to enable phenazine production. Products from strain *E. coli* BL21(DE3) expressing pT7-6A-G, which contains the entire biosynthetic gene cluster, consisted almost entirely of PCA, and the yield was nearly equal to that from wild-type *P. fluorescens* 2-79 (Fig. 4B). In contrast, strain BL21(DE3) expressing pT7-6CDEFG, which lacks *phzA* and *phzB*, produced a heterogeneous mixture of nitrogen-containing aromatic and heterocyclic compounds. Analysis of these compounds by mass spectrometry confirmed the presence of smaller amounts of PCA as well as of a mixture of heterocyclic, nitrogen-containing compounds including unsubstituted phenazine. Expression of other combinations of *phz* genes, including *phzAB*, *phzABCD*, *phzCD*, *phzCDE*, and *phzB* or *phzG*, yielded no detectable phenazine products (Fig. 4).

The *phzX* and *phzY* genes occupy the same relative position in the phenazine biosynthetic cluster of *P. aureofaciens* 30-84 as do *phzA* and *phzB* in the 2-79 gene cluster (Fig. 4C), and all four gene products are highly conserved (Fig. 2). To determine whether *phzXY* influences the quality and quantity of compounds produced from the phenazine biosynthetic cluster of strain 30-84, plasmids pT7-5X-D, containing the entire 30-84 biosynthetic cluster, and pT7-5FABCD, containing only the *phzFABCD* genes (Fig. 4C), were expressed in *E. coli* BL21(DE3). As with the cloned genes from 2-79, the complete gene cluster in pT-7X-D enabled the almost exclusive production of PCA. Plasmid pT7-5FABCD, lacking *phzXY*, synthesized smaller amounts of PCA and a mixture of other nitrogen-containing aromatic compounds.

DISCUSSION

Results of structural and functional analyses presented here show that products of the *phzABCDEFG* gene cluster are responsible for synthesis of PCA by *P. fluorescens* 2-79. Phenazines are products of the bacterial common aromatic amino acid pathway, with chorismate as the probable branch point intermediate (38), but the precise mechanism of synthesis and the identity of the biosynthetic intermediates remain unknown. Our evidence that the products of *phzC*, *phzD*, and *phzE* have significant homology to well-characterized enzymes of the shikimate and tryptophan biosynthetic pathways is consistent with previous findings (19, 38) and provides new insight into the phenazine biosynthetic pathway in fluorescent *Pseudomonas* spp.

PhzC shows a high degree of similarity to plant DAHP synthases that catalyze the first step of the shikimate pathway. In bacteria, DAHP synthase isoenzymes are regulated transcriptionally as well as through feedback inhibition by specific amino acids or pathway intermediates and thus represent a key control point regulating carbon flow into the shikimate pathway (16). All known DAHP synthases recently were grouped into two distinct classes based on protein sequence similarity. Our sequence data indicate that the PhzC protein from *P.*

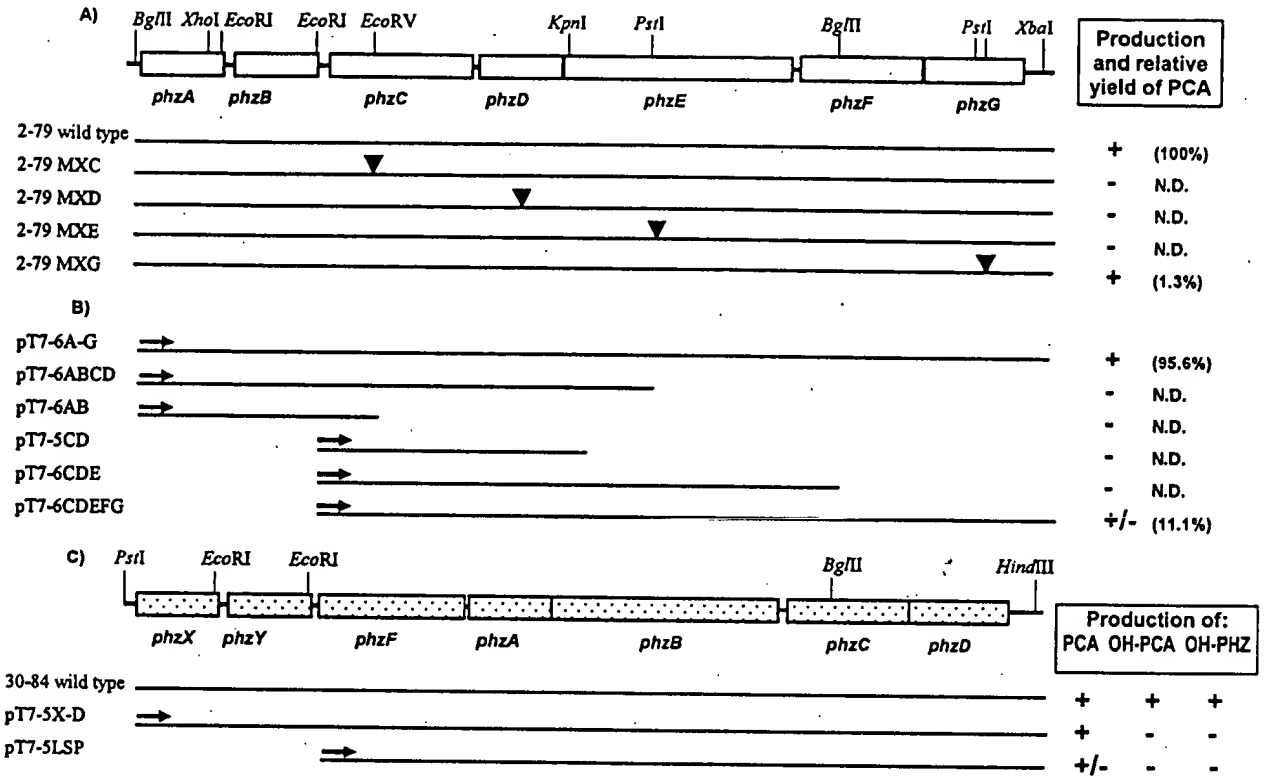

FIG. 4. Analysis of phenazine biosynthesis in *P. fluorescens* 2-79 gene replacement mutants (A) and by *E. coli* BL21(DE3) clones harboring plasmids with different sets of *phz* genes from *P. fluorescens* 2-79 (B) or *P. aureofaciens* 30-84 (C) cloned under the control of a T7 promoter. Restriction maps, locations of individual *phz* genes, and DNA fragments contained within plasmids used in the study are shown. Arrows indicate the position and orientation of the T7 promoter. Solid triangles denote locations of the Kan^r cassette insertions in the chromosome of *P. fluorescens* 2-79 mutants. N.D., not detected. OH-PCA, 2-hydroxyphenazine-1-carboxylic acid; OH-PHZ, 2-hydroxyphenazine.

fluorescens 2-79 is a typical type II enzyme, together with *P. aureofaciens* PhzF and the recently studied DAHP synthases from *Streptomyces coelicolor* and *S. rimosus* (39). Being expressed late in growth, PhzC could function to divert common carbon metabolites into the shikimate pathway, providing the high levels of chorismic acid needed to support the synthesis of PCA, which can accumulate in culture media at concentrations of up to 1 g/liter. The remainder of the enzymatic activities needed for chorismate biosynthesis probably are provided by shikimic acid pathway enzymes encoded by *aroD*, *aroB*, *aroE*, *aroL*, *aroA*, and *aroC*, since these genes are known to be expressed constitutively in pseudomonads (24).

Based on their sequence, it is likely that products of the *phzD* and *phzE* genes act to modify chorismate prior to the condensation reaction resulting in formation of the phenazine nucleus. The first two-thirds of the PhzE protein display relatively weak similarity to component I of bacterial anthranilate synthases. The C-terminal part (amino acid residues 438 to 637) exhibits strong homology to members of class I GATase enzymes, which comprise component II of anthranilate synthases. The PhzE protein is most closely related to a small subset of anthranilate synthases of unusual structure (TrpE from *Streptomyces venezualae*, TrpE from *Rhizobium meliloti*, and TrpE from *Azospirillum brasilense*) that have evolved as a fusion of genes encoding anthranilate synthase components I and II (2, 8). Component I of anthranilate synthase is a bifunctional enzyme that catalyzes the formation of the aromatic product anthranilate from chorismic acid in two discrete steps (22). The first step is catalyzed by aminodeoxyisochorismate (ADIC) synthase and is thought to involve amination of chorismic acid to ADIC. The second step, which is catalyzed by ADIC lyase, involves elimination of pyruvate and aromatization to form anthranilate. ADIC remains enzyme bound, but it has been demonstrated that a substitution of a single amino acid residue within component I of anthranilate synthase is sufficient to uncouple ADIC synthase and ADIC lyase activity (22). Interestingly, it previously was postulated that ADIC could be a potential precursor for the phenazine compounds iodinin and aminophenoxazinone, produced by cultures of *Brevibacterium iodinum* (30). Collectively, these observations and the rather weak similarity between the PhzE protein and most bacterial anthranilate synthases may indicate that PhzE functions specifically as an ADIC synthase (Fig. 5). The GATase domain might confer upon PhzE the ability to use glutamine as a source for the amination of chorismate. This is consistent with the data of Römer and Herbert, who demonstrated that the amide nitrogen of glutamine serves as the immediate source of nitrogen in the heterocyclic nucleus of phenazine compounds (30).

The product of the *phzD* gene shows a high degree of similarity to bacterial 2,3-dihydro-2,3-dihydroxybenzoate synthases (isochorismatases). The best-studied isochorismatase, EntB from *E. coli*, has a predicted M_r of 32,500 and is active as a pentamer in the isochorismatase reaction, catalyzing the hydrolysis of pyruvate from isochorismic acid (11). The presence of an isochorismatase analog in the *phz* locus might reflect the need for pyruvate hydrolase activity, rather than an ADIC lyase activity (which could be provided by anthranilate synthase), for modification of the immediate precursor of the phenazine nucleus. EntB actually is a bifunctional enzyme (of-

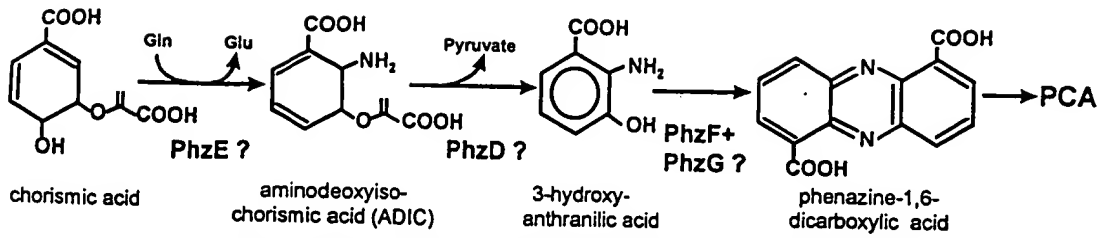

FIG. 5. Proposed action of PhzC, PhzD, PhzE, PhzF, and PhzG in the biosynthesis of PCA.

ten called EntB/G), with EntG activity encoded by the *entB* 3′ terminus (33). EntG functions as a part of the EntDEFG enterobactin synthetase multienzyme complex that catalyzes the last step of enterobactin biosynthesis. Interestingly, the PhzD protein resembles a truncated EntB, which lacks 78 amino acid residues at the C terminus. It therefore is probable that PhzD has only isochorismatase activity, which could function to remove the pyruvate side chain from ADIC to yield the putative phenazine precursor 3-hydroxyanthranilate (Fig. 5).

It seems likely that the products of *phzF* and *phzG* function in the condensation of two molecules of 3-hydroxyanthranilate, or a similar precursor, to generate the phenazine nucleus. Sequence analysis of PhzF revealed no motifs or similarities to other proteins of known function. However, PhzG resembles bacterial pyridoxamine-5′-phosphate oxidases that function in the de novo synthesis of pyridoxine (vitamin B_6) and in the conversion of pyridoxamine-5′-phosphate to pyridoxal phosphate, a cofactor for numerous transamination reactions (18). Interestingly, pyridoxal phosphate is required for aminodeoxychorismate lyase (PabC) activity in *E. coli* (14), where the cofactor is proposed to bind via an imine linkage to the 4-amino position of 4-amino-4-deoxychorismate during the synthesis of *p*-aminobenzoic acid (22). While it is tempting to speculate that pyridoxal phosphate may play a similar role in phenazine synthesis, binding at the 2-amino position of the hypothetical precursor 3-hydroxyanthranilate (Fig. 5), no conserved pyridoxal phosphate-binding motifs were identified in PhzF or, indeed, in any of the other *phz* gene products. Thus, the step(s) involved in this final condensation reaction remains obscure.

We also were unable to identify any motifs or similarity between products of *phzA* and *phzB* and protein sequences listed in various databases. However, PhzA and PhzB are remarkably similar to each other (Fig. 2), which strongly suggests that their cognate genes evolved as the result of a duplication event. Homologs of *phzA* and *phzB* also were identified in the phenazine biosynthetic locus from *P. aureofaciens* 30-84. Functional analysis indicated that in both 2-79 and 30-84, these genes influence the kinds and relative amounts of aromatic and heterocyclic compounds synthesized as a result of *phz* gene expression. Thus, *E. coli* expressing the complete *phz* locus synthesized large amounts exclusively of PCA whereas the same host expressing the *phzCDEFG* genes produced large quantities of a mixture of aromatic and heterocyclic nitrogen-containing compounds that included only minor amounts of PCA. PhzA and PhzB may stabilize a multienzyme phenazine biosynthetic complex, whose existence has been postulated previously (6). In their absence, the biosynthetic system still functions, but the specificity and perhaps also the efficiency decrease dramatically.

Results of this study indicate that the phenazine biosynthetic gene cluster from *P. fluorescens* 2-79 is remarkably conserved relative to that previously described from *P. aureofaciens*. In related work, we recently have shown that a portion of the pyocyanin biosynthetic locus from *P. aureofaciens* PAO1 retains certain of these same organizational and structural features (20). It thus appears that different fluorescent *Pseudomonas* species may have a common pathway, which confers upon them the ability to synthesize the phenazine nucleus.

How, then, can the specific array of phenazine compounds produced by individual species of fluorescent pseudomonads be explained? *P. fluorescens* 2-79 produces only PCA, whereas *P. aureofaciens* 30-84 produces, in addition to PCA, lesser amounts of 2-hydroxyphenazine-1-carboxylic acid and (probably by spontaneous, nonenzymatic decarboxylation) small quantities of 2-hydroxyphenazine (25, 27). The conversion of PCA to 2-hydroxyphenazine-1-carboxylic acid in strain 30-84 previously was attributed to the product of *phzC* (27), which has 93.5% identity and 95.3% similarity to the corresponding PhzF protein from *P. fluorescens* 2-79. Although small differences in sequence between these proteins cannot formally be ruled out as contributing to product specificity, the sequence data do not provide a useful insight into how *phzC* might function in derivatization of the phenazine nucleus, nor is the location of the gene within a cluster of core biosynthetic genes consistent with modification of the key product. Moreover, our observations that expression in *E. coli* of incomplete loci lacking *phzAB* or *phzXY* yielded mixtures of compounds including PCA and unsubstituted phenazine, whereas expression of the complete locus from either strain enabled the synthesis of large amounts of essentially homogeneous PCA, argue that the compounds detected in earlier expression studies with strain 30-84 that included only *phzFABCD* are products of inefficient or nonspecific synthesis that do not accurately reflect the biosynthetic potential of the intact locus. Our results obtained by HPLC, UV-visible spectral analyses, and mass spectrometry support the idea that the *phzF* product from strain 2-79 and its homolog from strain 30-84 participate directly in the condensation reactions leading to production of phenazine-1,6-dicarboxylic acid, the postulated precursor of other phenazines including PCA (19). We further hypothesize that enzymes encoded by other genes which may or may not be physically linked to the *phz* loci, as well as spontaneous chemical reactions, are responsible for modification of phenazine-1,6-dicarboxylic acid or PCA to form the various phenazine compounds characteristic of different species of fluorescent pseudomonads.

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